NEUROTRANSMITTER INVOLVEMENT IN EPISODIC LUTEINIZING HORMONE (LH) RELEASE IN ADULT OVARIECTOMIZED RATS

by

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"...We shall not cease from exploration
and the end of all our exploring
will be to arrive where we started
and know the place for the first time..."[1]

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[1] From "Little Gidding" in FOUR QUARTERS by T. S. Eliot,
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ABSTRACT

Luteinizing hormone (LH) release in ovariectomized (ovx) rats occurs in an episodic manner. Various studies have suggested that the brain may play a significant role in the origin of these episodic LH bursts. Central monoaminergic neurons have been implicated in the control of LH secretion by a number of investigators. Although norepinephrine has been suggested to play an excitatory and serotonin an inhibitory role in the neural events controlling the release of LH, the role, if any, of dopamine is not clear. This study was designed to investigate the possible involvement of these neurotransmitters in the release of LH releasing hormone that precedes pulsatile LH discharge in adult ovx rats.

Unrestrained, unanesthetized ovx rats with indwelling right atrial cannulae were bled continuously (30, 50, or 100 µl of whole blood / 4-6 minutes for 3-4 hours) and blood samples analyzed for LH by radioimmunoassay. Animals were treated with compounds reported to affect monoamine biosynthesis or act on adrenergic or dopaminergic receptors.

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In other individual rats, changes in the hypothalamic levels of norepinephrine (NE) and dopamine(DA) after drug administration were determined by a radioisotopic enzymatic catechol-o-methyltransferase assay.

Alpha-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor, significantly decreased hypothalamic norepinephrine (HNE) and hypothalamic dopamine (HDA) concentrations but failed to alter episodic LH release. Two dopamine-β-hydroxylase inhibitors (U-14,624 and FLA-63) caused marked reductions in HNE, small but not statistically significant increases in HDA, and an inhibition of episodic LH secretion. However, administration of dihydroxyphenylserine, a precursor of NE, and isoproterenol, a β-adrenergic receptor agonist, to animals pretreated with U-14,624 failed to restore episodic LH release patterns.

Apomorphine, a dopamine receptor agonist caused a transient (50-60 minutes) but marked inhibition of episodic LH release. Saline injection had no effect. Similar results (a marked inhibition of episodic LH release (2½-4 hours)) were obtained after the administration of ET495, a longer acting dopamine receptor agonist. Control injections of distilled water had no effect. To test whether the inhibitory action of apomorphine or ET495 was exerted by a specific action on DA receptors, ovx rats were pretreated before apomorphine with pimozide, a blocker of DA receptors, and before ET495 or apomorphine with d-butaclamol, another blocker of DA receptors. Pimozide or d-butaclamol alone did not alter episodic LH release but each prevented the inhibitory effects seen following apomorphine or ET495. L-butaclamol had no effect.

In order to relate any possible stress induced changes in plasma corticosterone levels to the inhibitory action of apomorphine or ET495 on LH release, plasma corticosterone levels were determined. Increases in plasma corticosterone levels were seen both following apomorphine or ET495 alone (when LH release was inhibited) as well as after apomorphine or ET495 administration to d-butaclamol pretreated rats (when LH levels did not change).

Biochemical and pharmacological studies suggest an interaction between serotonergic and catecholaminergic neurons in the brain. However, hypothalamic serotonin levels were not determined in the present studies after pretreatment with β-hydroxylase or tyrosine hydroxylase inhibitors or DA receptor agonists. However, the inhibitory effect of apomorphine on episodic LH release still occurred in ovx rats pretreated with parachlorophenylalanine (PCPA) an inhibitor of serotonin synthesis suggesting that its action may be independent of brain serotonin levels.

Thus, depletion of HNE by U-14,624 or FLA-63 inhibits episodic LH release suggesting that in adult ovx rats NE may play an excitatory role. Activation of DA receptors by apomorphine or ET495 also inhibits this process. However, the inability of pimozide or d-butaclamol alone to alter episodic LH discharge, suggests that under physiological conditions DA may not play a role in the modulation of episodic LH release.

To further examine the roles of various neurotransmitters in episodic LH release these substances were infused into the third ventricle of ovx rats. Control infusions of artificial cerebrospinal fluid (CSF) had no effect. The administration of DA-HCl in doses ranging from 2.5-10 μg/25λ CSF/h inhibited episodic LH release during the infusion period in 60% of the animals treated with 10μg DA/hour. This effect still occurred however in 43% of the animals pretreated with d-butaclamol. Infusion of L-norepinephrine bitartrate (NE) in doses ranging from 2.5-20 μg/25λ CSF/hour, inhibited pulsatile LH release during the infusion period. Lower doses (0.5 and 1 μg/25λ CSF/hour) had no effect. These results suggest either that DA may exert its inhibitory effect by first being transformed into NE, or that d-butaclamol could not bind to DA receptors around the third ventricle regions. The inhibitory effect on episodic LH release seen during NE administration into the third ventricle suggests that NE may have an inhibitory role in episodic LH release possibly by stimulating adrenergic receptors in that region, which exerts an inhibitory influence in the process. Finally, infusion of serotonin either inhibited episodic LH release during or even after the infusion period or had no effect.

To examine a possible interaction between estrogen and catecholmines in episodic LH release, ovx rats were primed with 5μg estradiol benzoate 100gbw/day for two days prior to peripheral administration of DA receptor agonists or central administration of NE into the third ventricle. Although the levels of LH were low after estrogen administration, apomorphine (1.50mg/kg sc) and ET495 (20mg/kg ip) failed to alter the blood levels of LH; NE infusion (5-20μg/25λ/hour) into the third ventricle increased the levels of LH in 50% of the animals after the infusion period. This suggests that the presence of estrogen may be capable of reversing the inhibitory effect of NE on episodic LH release in ovx rats.

In summary, these experiments show that 1) when NE synthesis is inhibited by peripheral administration of β-hydroxylase inhibitors to adult ovx rats, episodic LH release is inhibited. 2) when NE is administered centrally into the third ventricle of ovx rats, it inhibits episodic LH release, 3) the presence of estrogen may reverse the inhibitory effect on LH release seen during NE infusions in ovx rats. 4) activation of DA receptors is capable of inhibiting episodic LH release and this effect is not exerted via a stress induced release of adrenal corticosterone.

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UMI

TABLE OF CONTENTS

Page

Abstract i

Acknowledgements vi

List of Figures ix

Introduction 1

I. Review of the literature 1
  A. Neural control of luteinizing hormone (LH) release 1
  B. Localization of luteinizing-hormone-releasing hormone (LH-RH) 9
  C. Episodic LH release 12
    1. Evidence for its presence 12
    2. Site of origin 15
    3. Factors that effect this pattern of release 18

II. Brain Neurochemistry 21
  A. Introduction 21
  B. Distribtion of central catecholaminergic neurons 23
    1. Dopamine pathways 23
    2. Norepinephrine pathways 24
    3. Epinephrine pathways 27
  C. Distribution of central serotonergic neurons 27

Page
D. Monoamine metabolism 28
  1. Catecholamine metabolism 29
    a. Synthesis 29
    b. Storage 31
    c. Release 34
    d. Receptor interaction-post-synaptic action of catecholamines 34
    e. Reuptake-inactivation 35
  2. Serotonin (5-HT) metabolism 36
E. Experimental approaches to study brain amine function 38

III. Monoamine, Involvement In Luteinizing Hormone (LH) Release 39

Statement of Purpose 42

Materials and Methods 46

Results 58
  I. Administration of drugs affecting catecholamine or serotonin biosynthesis or acting on adrenergic or dopamine receptors 58
  II. Neurotransmitter infusion in the third ventricle 80
  III. Estrogen administration 88

Discussion 93

References 104

List of Figures

Page

1. Patterns of episodic LH release in two ovariectomized rats. 59

2. Effect of aMPT (250mg/kg ip) on episodic LH release in an ovx rat. 60

3. Effects of U-14,624 and FLA-63 on episodic LH release in individual ovx rats. 62

4. Effect of saline (stippled bars) or aMPT (250 mg/kg ip; open bars) given three hours earlier on hypothalamic levels of norepinephrine and dopamine in individual ovx rats. 63

5. Effect of U-14,624 and FLA-63 on hypothalamic levels of norepinephrine and dopamine in individual ovx rats. 64

6. Effect of dihydroxyphenylserine (DOPS; 250mg/kg ip) on LH release in an ovx rat pretreated 18 hours earlier with U-14,624 (200 mg/kg ip). 66

7. Effect of apomorphine on episodic LH release in individual ovx rats. 67

8. Effect of saline injection (SC) during experimental bleeding period on episodic LH release in an ovx rat. 69

9. Effect of ET495 (20mg/kg ip) on episodic LH release in individual ovx rats. 70

10. Effect of distilled water injection (ip) during experimental bleeding period on episodic LH release in individual ovx rats. 71

11. Effect of apomorphine (APO; 1.5mg/kg/sc) on episodic LH release in individual ovx rats pretreated 16 hours earlier with pimozide (1.26mg/kg sc). 72

12. Effect of apomorphine (APO; 1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with d-butaclamol (1mg/kg ip). 74

Page

13. Effect of ET495 (20mg/kg ip) on episodic LH release in individual ovx rats pretreated 3-4 hours earlier with d-butaclamol (1mg/kg ip). 75

14. Effect of apomorphine (APO; 1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with d-butaclamol (0.5mg/kg ip). 76

15. Effect of ET495 (20mg/kg ip) or apomorphine (1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with L-butaclamol (1mg/kg ip). 77

16. Effect of ET495 or apomorphine alone (left) or after d-butaclamol pretreatment (right) on plasma corticosterone levels in ovx rats. 79

17. Effect of apomorphine (1.5mg.kg sc) on episodic LH release in individual ovx rats pretreated 70 hours earlier with pCPA (32mg/100g bw ip). 81

18. Effect of cerebrospinal fluid (25λ containing 1λ 0.1% ascorbic acid) infusion into the third ventricle on episodic LH release in individual ovx rats. 82

19. Effect of dopamine-HCl (10μg/25λCSF/h) infusion into the thrid ventricle on episodic LH release in individual ovx rats. 83

20. Effect of dopamine-HCl (10μg/25λCSF/h) infusion into the third ventricle on episodic LH release in individual ovx rats pretreated 4-5 hours earlier with d-butaclamol (1mg/kg sc). 85

21. Effect of L-norepinephrine bitartrate (10,20, or 50μg 25λ CSF/h) infusion into the third ventricle on LH release in individual ovx rats pretreated 18 hours earlier with U-14,624. 86

22. Effect of L-norepinephrine bitartrate (20μg/25λ CSF/h in the top and 10μg/25λ CSF/h in the bottom) infusion into the third ventricle, on episodic LH release on individual ovx rats. 87

23. Effect of serotonin (10μg/25λ CSF/h) infusion into the third ventricle, on episodic LH release in individual ovx rats. 89

24. Effect of apomorphine (APO; 1.5mg/kg sc) or ET495 (20mg/kg ip) on LH release in individual ovx rats primed for two days prior with estradiol-benzoate (5μg/100g bw/day sc in oil). 90

UMI

Page

25. Effect of L-norepinephrine bitartrate (5, 10, 20μg/25λ CSF/h) infusion into the third ventricle on LH release in individual ovx rats primed for two days prior with estradiol benzoate (5mg/100g bw/day sc in oil). 92

INTRODUCTION

I. Review of the Literature

A. Neural Control of Luteinizing Hormone (LH) Release

The existence of hypothalamic substances regulating anterior pituitary function was postulated many years ago (Green and Harris, 1947) and during the past few years, much progress has been made to support this hypothesis. It is generally accepted now that anterior pituitary function is controlled by the central nervous system (CNS) with the hypothalamus exerting an important role. That this control is not mediated by nerve fibers, was suggested by the absence of an appreciable nerve supply to the anterior pituitary gland (Harris 1955). A portal system of blood vessels between the median eminence (ME) and pituitary appeared as the likely pathway for the hypothalamic regulation of pituitary functions. Thus, Green and Harris formulated the so called "neurovascular hypothesis of adenohypophysial regulation". They assumed that the hypothalamus produces substances which, after being released in the ME, enter the portal circulation and therein are carried to the pituitary.

The demonstration of the existence of a corticotropin-releasing factor (for review Schally, et al., 1973) opened the way for subsequent discoveries of other hypothalamic regulatory substances. Overwhelming evidence is now available to support the concept that the neural impulse for the discharge of LH from the anterior pituitary gland is mediated by a hormonal substance - luteinizing hormone - releasing hormone (LH-RH), which originates in the hypothalamus, and perhaps in some extrahypothalamic areas as well and reaches the pituitary through the hypophyseal portal system (Schally, et al., 1973). Investigators utilizing the techniques of electrical stimulation of the hypothalamus, lesions of discrete areas of the CNS, transplantation of the pituitary, interruption of neural connections of the medial basal hypothalamus (MBH), and implantation of sex steroids, obtained data that the hypothalamus is the main central nervous component in the control of the gonadotropin secretion.

In addition, it has been known for many years that reproduction is influenced by seasonal, environmental and emotional factors. Two classes of factors that influence gonadotropin secretion may be referred to as "open loop" and "closed loop" factors. "Open loop" involves stimuli from both the external environment (e.g. light, sexual stimuli, etc.) and the internal environment (e.g. state of nutrition, maturational factors, etc.). "Closed loop", or feed-back control, involves regulation predominantly by circulating levels of gonadal steroids. One can moreover distinguish between positive (stimulatory) and negative (inhibitory) feed-back control (Davidson, 1969).

Considerable evidence has been collected on the stimulatory feed back effects of both estrogenic and progestational steroids. It is well established now that estrogen or progesterone may advance the appearance of ovulation during normal estrous cycles in the adult rat. Similarly, these steroids can advance ovulation in cows, rabbits, monkeys, and women (Everett, 1964). Moreover, ovarian steroids have produced precocious stimulation of gonadotropic function in prepuberal animals (Rennels and O'Steen, 1967; Meyer and McCormack, 1967; Grayburn and Brown-Grant, 1968; Gallo and Zarrow, 1968). Ovulation can also be stimulated by steroids during the normally quiescent period of pregnancy in rats (Brown-Grant, 1969). Success in inducing ovulation in pregnant rats depends on precise timing of the estrogen injection during the early days of pregnancy (Everett, 1948; Brown-Grant, 1969). Brown-Grant (1969) showed that exogenous progesterone inhibited (or delayed) ovulation when administered at any stage of the rat estrous cycle, except from the time of the presumed onset of estrogen secretion until the time of ovulatory release of LH during which period the effect was stimulatory. The stimulatory effect of progesterone in immature rats also depended on previous exposure to endogenous or exogenous estrogen (Grayburn and Brown-Grant, 1968). The stimulatory phase of progesterone's effects on ovulation in the rabbit (Kawakami and Sawyer, 1967) is also a function of high pre-existing levels of estrogen. Thus, the ratio between circulating estrogen and progesterone concentrations appear to be a main factor for the appearance of either the stimulatory effect or inhibitory effect of the ovarian steroids on pituitary function.

In order to localize the site(s) of the stimulatory effect of ovarian steroids, studies using lesions of certain areas of the central nervous system (CNS) (Quinn and Zarrow, 1964; Barraclough et al., 1964; Bishop et al., 1972) electrochemical and electrical stimulation of the preoptic region of the brain during the estrous cycle, (Cramer and Barraclough, 1971; Clemens, et al., 1971; Kalra, et al., 1973; Turgeon and Barraclough, 1973; Kalra and Kalra, 1974), and intracranial implantation of sex steroids in different CNS regions (Davidson and Sawyer, 1961; Döcke and Dörner, 1965; Ramirez and Sawyer, 1965; Smith and Davidson, 1968) have shown that there are two sites of stimulatory feed-back action, the anterior hypothalamic - medial preoptic region and the pituitary. Moreover, experiments in which partial deafferentation of the medial basal hypothalamic region of the rat was performed (Halász and Gorski, 1967), showed that neural afferents which enter the MBH anteriorly are necessary for the ovulatory surge of LH from the pituitary. Recent evidence supports the hypothesis that steroids can exert direct effects on the pituitary gland. These results were obtained from studies measuring the pituitary responsiveness to exogenous LH-RH during the estrous cycle (Arimura et al., 1972; Debeljuk, et al., 1972), or after exogenous administration of different amounts of estrogen and progesterone in intact and castrated rats (Debeljuk, et al., 1972; 1974; Greeley, et al., 1975). For example, in cyclic ewes, the same dose of LH-RH induces a greater change in the concentration of LH in the serum when administered on the day of the onset of estrus (when estrogen levels are high) than when it is administered at any other stage of the estrous cycle (Reeves, et al., 1971). When a similar experiment was performed in golden hamsters and rats, the greatest response to LH-RH was observed during proestrus (Schally, et al., 1973). Exogenous LH-RH also induced the release of highest concentrations of LH at midcycle in rhesus monkeys and normal women (Schally, et al., 1973). Thus the concentration of circulating steroids could alter the sensitivity of the pituitary gland to LH-RH, and the ovulatory surge of LH release may be caused in part by the greater sensitivity of the pituitary gland to LH-RH, in addition to an increased release of endogenous LH-RH occurring at that time.

A variety of experimental approaches have yielded results which have indicated that the hypothalamus is also the site of the inhibitory feed-back action of gonadal steroids. The pituitaries of female rats with a neurally isolated MBH responded to castration; pituitary LH release increases and castration cells are found in the anterior pituitary (Halász and Gorski, 1967; Mavić and Nikitovitch-Winer, 1967). Estrogen implants in the MBH result in ovarian atrophy (Lisk, 1960; Davidson and Sawyer, 1961), prevent the formation of castration cells in the pituitary of the spayed rat (Lisk, 1963) and rabbit (Kanematsu and Sawyer, 1963), and inhibit the post-castration rise in pituitary and plasma LH (Ramirez, et al., 1964; Kanematsu and Sawyer, 1964). Smith and Davidson (1974) were able to lower circulating LH levels in ovx rats with estrogen implants in the MBH which did not alter the pituitary threshold to exogenous LH-RH. Ferin, et al. (1974) also have shown that the intrahypo-

UMI

thalamic estrogen-responsive sites influencing LH secretion in the female rhesus monkeys are located in the mediobasal hypothalamus, suprachiasmatic, infundibular, and ventromedial nuclei and the mammillary complex. Administration of exogenous estrogen inhibited the post-castration rise of LH release and episodic LH release in ovx rats (Blake, et al., 1974) and monkeys (Krey, et al., 1975) as well as in ovx rats and monkeys with hypothalamic deafferentation (Blake, et al., 1974; Krey, et al., 1975). However, in female rats, estrogen implants have been found effective in inhibiting gonadotropin secretion when placed either in the pituitary or the median eminence region (Ramirez, et al., 1964; Chowers and McCann, 1976, Davidson 1969). Moreover, it has been found that peripheral administration of estrogen initially inhibits LH release with an associated decrease in pituitary sensitivity to exogenously administered synthetic LH-RH (Negro-Vilar, et al., 1973; Blake, et al., 1974). However by 8 hours after estrogen injection, the responsiveness of the gland to LH-RH is enhanced - a biphasic effect - (Libertun, et al., 1974).

Recently, using diestrous rats in which normal ovulation was blocked by progesterone, Terasawa and Kawakami (1974) induced ovulation with either systemic injections of estrogen or estrogen implants in the medial amygdala or bed nucleus of the stria terminalis. Cutting the stria terminalis blocked this stimulatory feed-back action of estrogen implants in the amygdala. The same investigators showed that electrical stimulation of the dorsal hippocampus inhibited the increase in multi-unit activity occurring in the medial preoptic area and arcuate nucleus following estrogen implants in the medial amygdala on the second day of diestrus in 5-day cycling rats. Thus, extra hypothalamic areas such as the amygdala and hippocampus may be involved in either facilitatory or inhibitory influences on gonadotropin release. Moreover, results from several laboratories using scintillation counting of dissected brain areas after administration of radioactive estradiol, autoradiographic techniques, and nuclear isolation procedures, agree that estradiol is concentrated by cells of the medial preoptic area, medial anterior hypothalamus, ventromedial hypothalamic nucleus, arcuate nucleus, ventral premamillary nucleus, medial and cortical nuclei of the amygdala, hippocampus, olfactory tubercle, the lateral septum and the bed nucleus of the stria terminalis (Pfaff and Keiner, 1973). Thus, it is of interest to note that there is a high degree of anatomical correspondence between sites of highest estrogen uptake and areas supposedly concerned with the control of estrogen dependent functions.

In conclusion, gonadotropin release is controlled by a region which extends from the medial preoptic area rostrally through the anterior hypothalamus to the arcuate median eminence region caudally. The most rostral portions of this region, namely, the suprachiasmatic, medial preoptic and anterior hypothalamic areas, appear to be involved in the preovulatory release of luteinizing hormone and in mediating the stimulatory effects of gonadal steroids, which aid in provoking the preovulatory discharge. The more caudal portions of the gonadotropin - controlling region, that is the median eminence - arcuate region, appear to be involved in mediating the negative feed-back of gonadal steroids. Both negative as well as positive feed-back also appear to operate directly on the anterior pituitary as evidenced both by studies of estrogen implantation in the pituitary gland and by the ability of estrogen to modify the pituitary responsiveness to exogenously administered synthetic LH-RH.

The existence of a "short" feed-back mechanism for the control of LH secretion, in which the controlling signals are the pituitary hormones themselves, was proposed by Sawyer and Kawakami (1959) and by Kawakami and Sawyer (1959) on the basis of the observation that, in estrogen primed ovx rabbits, the EEG was influenced by exogenous gonadotropins or by endogenous gonadotropins released by coitus. Implants of LH in the ME suppress LH secretion in normal or castrated rats (Corbin and Cohen, 1966; Corbin 1966) or in immature animals (Ojeda and Ramirez, 1969), and vaginal estrous cycles in normal adult female rats became irregular (Corbin and Cohen, 1966). The participation of the hypothalamus in the "short" feed-back effect of LH is also indicated by the observation that hypophysectomy induces elevations in plasma LH-RH activity (Nallar and MaCann, 1965; Pelletier, 1965) and exogenous LH reduces this LH-RH activity (McCann, et al., 1968). The electrophysiological approach to the study of the effects of LH on the brain helped to define more precisely the type of the responses evoked by the exogenous administration of LH. For example, Terasawa and Sawyer (1968), showed that the hormone activates the neurons of the arcuate region and inhibits those of the anterior hypothalamic-medial preoptic area.

Much more work is obviously still needed in this field to elucidate the existence of and the actual mechanism of action involved in "short" feed-back signals and their possible significance in the control of pituitary function.

B. Localization of Luteinizing Hormone-Releasing Hormone (LHRH)

Since the demonstration of LHRH activity in hypothalamic extracts (McCann, *et al.*, 1960), numerous investigators have attempted to define anatomically the site(s) of LHRH synthesis. Until recently, one method used to determine the localization of LHRH was based on the release of LH from pituitaries incubated in vitro with extracts of brain sections. The results indicated that LH-releasing activity of rat brain was for the most part concentrated in the medial basal hypothalmus (MBH) but also was present to a lesser extent in more rostral preoptic-suprachiasmatic tissue (McCann, 1962; Crighton *et al.*, 1970; Quijada, *et al.*, 1971). More recently, the isolation, characterization and subsequent synthesis of sufficient quantities of LHRH to permit its use as an antigen (or hapten) has enabled workers attempting to localize LHRH to use sensitive radioimmunoassay and immunohistochemical techniques. With the availability of the microdissection method of Palkovits (1973) in combination with radioimmunoassay, regional distribution of LHRH has been better determined. Thus, two major methods have been used: a) microdissection of brain tissue followed by extraction of the LHRH and analysis by radioimmunoassay, b) immunohistochemical localization. Using the former approach, Palkovits and coworkers (1974) found LHRH in the arcuate nucleus (3 ng/mg protein) and median eminence (ME) (29 ng/mg protein) of the rat. Other hypothalamic nuclei - the ventromedial, supraoptic and suprachiasmatic nuclei - have small amounts. Wheaton, et al., (1975) found LHRH to be present in the medial preoptic area in the region co-extensive with the organum vasculosum of the lamina terminalis (OVLT). Studies by Kizer, et al. (1976), using the microdissection technique, have confirmed the presence of LHRH in the OVLT (14 ng/mg protein). Brownstein, et al. (1976) found that LHRH does not seem to be in the same medial basal hypothalamic cells as dopamine, since selective destruction of catecholaminergic neurons by 6-hydroxydopamine administered into the third ventricle of the rat, caused an 80%-85% decrease in dopamine and norepinephrine in the arcuate nucleus and median eminence but no change in LHRH levels. Moreover, in an elegant study, Weiner et al. (1976) showed that the cell bodies of neurons producing the LHRH found in terminals in the OVLT lie outside the MBH. Moreover, at least a portion of the LHRH neurons terminating in the ME may have their cell bodies within the MBH, since significant LH-RH content, as assessed by radioimmunoassay and immunochemistry, was still found 11 days after complete deafferentation of the MBH.

The results obtained form immunostaining have varied from species to species. In a series of papers, Barry and his collaborators(Leonardelli, et al, 1973; Barry and Dubois, 1974; Barry et al., 1973; Barry et al., 1974) working with the guinea pig, observed immunoreactive cells in the anterior and MBH. Many fibers were also visible and these investigators postulated a preoptico -

infundibular LRF neurosecretory pathway giving off numerous collaterals which end in the ME around the capillaries of the primary portal plexus (Barry and Dubois, 1974). Barry et al. (1974) described LHRH-containing cells in the anterior commissure parolfactory cortex, and immunoreactive endings in the amygdaloid nuclear complex. Hökfelt and associates reported LHRH containing fibers in the amygdala. The most consistent finding in a variety of mammals has been the concentration of LHRH in the zona externa of the ME (Barry, et al., 1973; Baker, et al., 1974; King, et al., 1974; Kordon, et al, 1974; Hökfelt, et al., 1975; Kozlowski, et al., 1975; Sétaló, et al., 1975). By both light and electron microscopy LHRH-containing fibers are also seen in the zona interna and in the subependymal lining of the floor of the third ventircle (Kordon, et al., 1974; Goldsmith and Ganong, 1975; Kordon and Ramirez, 1975). Two immunoelectron microscopic studies have now located LHRH in granules (750 to 950 A) within axon terminals near the portal capillaries in the rat (Goldsmith and Ganong 1976; Pelletier, et al., 1974).

Zimmerman, et al. (1974) found LHRH-positive perikarya only in the arcuate nucleus in mice. Immunoreactive deposits were also seen in the OVLT and zona externa of ME. However, in the mouse the majority of LHRH in the ME was present in tanycytes rather than axons, supporting the hypothesis that LHRH could be secreted into the CSF of the third ventricle and carried to the tanycytes in the floor and lower walls of the ME and then transported to portal blood (Scott, et al., 1974). The OVLT is the subject of considerable in-

UMI

terest at present as both immunohistochemical and regional assay approaches have localized LHRH in or near this structure, which lies in the middle of the preoptic area. Immunoreactive LHRH was found in the OVLT of the mouse, guineapig and rat (Zimmerman, et al. 1974; Barry, et al., 1974; Weiner et al., 1975). Large amounts of LHRH have been reported in the pineal gland of different animals (White, et al., 1974; Kozlowski and Zimmerman, 1974). Further studies are needed to establish the importance of LHRH in the OVLT region and pineal gland as well as the presence of LHRH-containing neuron in different brain regions.

C. Episodic Luteinizing Hormone (LH) Release

1. Evidence for its presence

a. In ovariectomized animals and hypogonadal and agonadal humans.

Interruption of the inhibitory effect of gonadal steroids on pituitary gonadotropin secretion results in a marked increase in plasma LH and FSH levels (Gay and Midgley, 1969; Atkinson, et al. 1970; Dierschke, et al., 1970; Yamamoto, et al., 1970). This rise in blood gonadotropin levels has been attributed in large part to the increased activity of the hypophysiotropic area of the hypothalamus, i.e. medio basal hypothalamus (MBH), and it is apparently due to the absence of the inhibitory signal exerted by gonadal steroids (McCann, 1969). Considerable evidence also indicates that the secretion of gonadotropins (particularly LH) in castrated

UMI

animals in which the negative feed-back system controlling "tonic" LH secretion has been interrupted, occurs in rhythmic, pulsatile discharges from the pituitary (Atkinson, et al., 1970; Dierschke, et al., 1970; Gay and Sheth, 1972; Butler, et al., 1972; Diekman and Malven, 1973).

The results from Gay and Midgley (1969) indicated that the concentration of LH in the peripheral plasma of the female rat might not increase until 2 to 3 days after ovariectomy. However, Tapper, et al. (1971) have shown that the changes in plasma LH concentration during the first few days after ovariectomy in the rat differ according to the stage of the cycle at which the operation is performed. When done on metestrus or estrus, no increase is seen for three or four days, whereas if done on diestrus, plasma LH levels are elevated by 8 hours following ovariectomy. This rapid rise following ovariectomy on diestrus was also seen by Yamamoto, et al. (1970). Gay and Sheth (1972) found that plasma LH concentrations in ovariectomized (ovx) rats were consistently elevated at least 20-fold over levels found in normal diestrous females, and appeared to be maintained by periods of LH release generally occurring at intervals of 20-40 minutes. Similar pulsatile patterns of LH release in ovx rats were reported by BLake and Sawyer (1974). Although it remains to be determined if LH release in intact rats demonstrates a similar periodicity, Gay and Sheth (1972) found that the preovulatory peak of serum LH in the proestrous rat is not composed of smaller periodic increases in serum LH. However, the ovulatory surge of LH in humans is composed of a number of oscillations in LH, whose magnitude appears to decrease as the midcycle surge is completed (Midgley and Jaffe, 1971).

Similar patterns of LH release, occurring at intervals of approximately one hour, have been observed in ovx monkeys (Dierschke, et al., 1970). Such circhoral oscillations did not occur in intact females (Dierschke, et al., 1970). Atkinson, et al. (1970) showed that in most ovx monkeys, the elevation in plasma LH levels occurred within one or two days after gonadectomy, and the mean plasma LH concentrations three weeks after gonadectomy represented an approximate 10-fold increase in the "basal" plasma level of LH observed in normal female and male monkeys. As in ovx monkeys and rats, circulating LH levels in ewes rise abruptly from nadir to peak at hourly intervals and appear to decline exponentially until the next pulsatile release (Butler et al., 1972; Diekman and Malven 1973). Large positive cranial outputs of LH (the jugular vein level minus the carotid artery level) were observed in association with peaks in arterial LH, indicating that these arterial peaks were due to pituitary release and not caused by changes in LH metabolism or degradation superimposed on a tonic rate of pituitary release (Butler, et al., 1972).

Moreover, rhythmic oscillations of LH release having an average interval of 1-2 hours were found in hypogonadal children (Kelch, et al., 1973) agonadal and hypogonadal adolescents and adults (Root, et al., 1972; Santen and Bardin, 1973) and in postmenopausal women (Yen, et al., 1972).

UMI

b. Intact animals and normal humans

Episodic LH release occurs even when the gonads are functionally present. Pulsatile release has been observed in intact bulls (Katongale, et al., 1971), rams (Bolt, 1971), sheep during the luteal phase of the estrous cycle (Braid, et al., 1976), normal men (Nankin and Troen, 1971; Boyar, et al., 1972; Rubin, et al., 1972; Santen and Bardin, 1973) and women during the different stages of the menstrual cycle (Midgley and Jaffe, 1971; Yen et al., 1972; Naffolin, et al., 1973; Santen and Bardin, 1973; Seyler and Reichlin, 1973). Episodic release of LH has been detected in domestic intact adult cockerels and gonadectomized fowl, with peaks occurring approximately every 1½ hours (Wilson and Sharp 1974).

2. Site of origin

The control mechanisms underlying these pulsatile discharges of LH from the anterior pituitary gland still remain elusive.

The rhythmic pulsatile discharges observed in ovx animals, in agonadal and hypogonadal subjects as well as in intact animals and normal men and women during the menstrual cycle, might suggest a "short-loop" feed-back regulation in which high circulating levels of LH drop below a certain level at which time a new surge is triggered (Motta, et al., 1969). However, there was no correlation between the time intervals of LH peaks and half-life measurements of LH in different animals. That the "short-loop" feed-back regulation is not the case is supported 1) by data which indicates the persistence of pulsatile gonadotropin-releasing hormone (GnRH) release after disconnection of the pituitary gland from the hypothalamus (Carmel, et al., 1976) and 2) by experiments in which continuous infusion of exogenous LH did not modify the pulsatile pattern of LH release in ovx monkeys (Knobil, 1974). Moreover, Osland, et al., (1975) found that when anterior pituitary fragments from ovx rats were superfused with a medium containing LH-RH for short intervals (2-5 minutes), these fragments could release LH in an episodic pattern, lasting for 30-40 minutes. Superfusion with constant levels of synthetic LH-RH produced a nonepisodic pattern of LH release. Moreover, Seyler and Reichlin (1974) found an episodic release pattern of LH-RH in human blood, suggesting that episodic secretion of LH-RH may cause episodic LH release from the pituitary. Supportive evidence for a central neuronal control of pituitary episodic LH release was obtained in experiments demonstrating a pulsatile LH-RH secretion from synaptosomes of the rat MBH (Bledsoe and Ramirez, 1976), as well as from studies indicating that pulsatile release is suppressed by centrally acting neuroleptic drugs and alpha adrenergic blocking agents (Bhattacharya, et al., 1972) and by administration of nicotine (Blake, et al., 1974).

From experiments using deafferentation techniques, one may conclude that the control of episodic LH release must reside in the mediobasal hypothalamus, since anterior or complete deafferentation of this area in ovx monkeys (Krey, et al., 1975) did not alter pulsatile LH release. However, episodic LH release persists in ovx rats only if deafferentation causes persistent estrus, not if persistent diestrus develops (Blake and Saywer, 1974).

It is well established that the negative feed-back effect of estrogen is exerted in the MBH in cycling animals (Davidson, 1969). However, the peripheral administration of estrogen in rats (Blake, et al., 1976), sheep (Diekman and Malven, 1973) ovx monkeys (Krey, et al., 1976), or the central injection of estrogen through cannulas stereotaxically implanted into hypothalamic sites (Ferin, et al., 1974), blocked the postovariectomy increase in LH and eliminated the pulsatile rhythm. Moreover, ovx rats as well as ovx monkeys with neurally isolated MBH (Blake, et al., 1974; Krey, et al., 1974) showed responses similar to intact animals with respect to the negative feed-back effects of peripherally administered estrogen. More recently, Plant, et al. (1976) reported that lesions in the arcuate-dorsal median eminence region in ovx adult monkeys reduced tonic LH secretion. Thus, the sites of the central neuronal components which regulate pulsatile LH secretion in the rhesus monkey and rat may be resident within the MBH-hypophysial unit.

Finally, it is of interest to note that in normal human subjects, Yen, et al. (1973) suggested that ovarian steroids can modulate the frequency and magnitude of the periodic release of LH by the anterior pituitary. This statement was based on qualitative and quantitative differences in pulsatile patterns of LH seen between different phases of the menstrual cycle and between subjects with or without ovarian function.

3. Factors that affect this pattern of LH release

a. Time of day

Plasma LH concentrations do not increase in ovx rats during the afternoon or evening hours; the episodic increases in circulating LH remain constant throughout the day (Gay and Sheth, 1972; Blake and Sawyer, 1974; Blake, 1974; Gallo and Osland, 1976). No diurnal variations of pulsatile LH release were observed in ovx monkeys (Knobil, 1974).

b. "Stress"

Blake (1975) reported that immobilization inhibited pulsatile LH release in ovx rats. Sham ovariectomy, leg break, injection of ACTH, or injection of corticosterone had no effect on the pulsatile rhythms of LH release.

c. Estrogen and progesterone

Peripheral administration of estrogen in ovx sheep (Diekman and Malven, 1973), rats (Blake, *et al*., 1974), or monkeys (Krey, *et al*., 1975) with deafferented MBH, inhibited pulsatile LH release and depressed plasma LH concentrations. Similar effects were observed after hypothalamic injections of estrogens through intracranially implanted cannulae. The most responsive hypothalamic site was the MBH. Thus, the main negative feed-back action of estrogen is exerted in the MBH. However, part of this feed-back may be exerted at the pituitary level as well, since injections of estrogen had a partial inhibitory effect on pituitary LH release after the exogenous administration of synthetic LH-RH to ovx rats

(Blake, *et al.*, 1974). Administration of progesterone, however, failed to alter the pulsatile rhythm or to lower plasma LH levels in ovx rats (Blake, *et al.*, 1974) or ovx monkeys unless massive doses were given (Yamiji, *et al.*, 1972), suggesting that estrogen may be the principal participant in the negative feed-back control of tonic LH secretion in primates and rats.

It is of interest to note that following an initial inhibition of episodic LH release due to the administration of estrogen to ovx monkeys, or to ovx monkeys with complete MBH disconnection, a gonadotropin surge occurs (positive feed-back action of estrogen). Drugs which were previously shown to be effective in inhibiting pulsatile LH release (eg. a-adrenergic blocking agents-phenoxybenzamine and phentolamine, and neuroleptic drugs-chloropromazine or haloperidol) failed to block this estrogen-induced LH surge in ovx monkeys (Knobil, 1974). In long-term ovx rats, administration of estrogen in very high doses (50 µg) or in combination with progesterone can exert a similar positive feed-back, i.e. LH surge (Legan, *et al.*, 1973).

d. *Other drugs*

Drugs capable of blocking ovulation in cycling rats, such as nicotine, urethane, phenobarbital (Blake, 1974) inhibited episodic LH release in ovx rats with or without deafferented MBH, suggesting that their action is exerted via a blockade of LH-RH synthesis or release from the MBH (Blake, 1974). Moreover, continuous deep ether anesthesia, injection of ethanol or pentobarbital (which blocked ovulation) caused marked but temporary inhibition of pulsalite LH release. None of these treatments inhibited the pituitary LH release in response to injection of synthetic LH-RH, suggesting their effect was not at the level of the pituitary. Pentobarbital was ineffective in blocking pulsatile LH release in ovx monkeys (Knobil, 1974).

e. Monoamine involvement in episodic LH release

Few studies have explored brain catecholamine involvement in episodic LH release. Bhattacharya, et al. (1972) have reported that a-adrenergic blocking agents (phenoxybenzamine or phentolamine) and neuroleptic agents (haloperidol and chlorpromazine) blocked episodic LH release in ovx monkeys. Beta-adrenergic blockade, in contrast, was without effect on pulsatile LH release in monkeys (Bhattacharya, et al., 1972). The effectiveness of a-adrenergic receptor blockade in inhibiting episodic LH release suggests catecholamine involvement in this process. However, episodic LH release persists in certain instances following complete deafferentation of the MBH in ovx rats (Blake and Sawyer, 1974), although the norepinephrine content of the deafferented hypothalamus is depleted by this procedure (Weiner, et al., 1972). This would suggest that norepinephrine may not play a role in episodic LH release. Recent studies (Gallo and Osland, 1976) show that electrical stimulation of the arcuate nucleus (ARH) in ovx rats inhibited episodic LH release. Stimulation in other hypothalamic areas outside the ARH was ineffective. Estrogen administration to the animals reversed the inhibitory effect on episodic LH release produced by electrical stimulation of the ARH. Moreover, more recent studies (Gallo, 1976) suggest a possible involvement of serotonin in mediating the inhibitory effect on episodic LH release in ovx rats produced by electrical stimulation of the arcuate-nucleus in the hypothalamus.

II. Brain Neurochemistry

A. Introduction

The cellular organization of the brain and spinal cord has been studied for many decades by classical histological and silver impregnation techniques. The initial demonstration that catecholamines are present in the central nervous system (CNS) was thought to be their involvement in sympathetic innervation of the vasculature of the brain (Von Euler, 1946). Biochemical studies of Vogt (1954) gave the first evidence to suggest that noradrenergic neurons exist within the CNS. They found a non-uniform distribution of norepinephrine in the cat brain with highest concentrations in the area postrema and areas associated with the autonomic system. Cervical sympathectomy did not affect the catecholamine content of those areas. This was an important indication that noradrenergic nerve cell bodies may be within the brain. Biochemical determination of serotonin in dog brain showed a distribution similar to that of norepinephrine (Amin, _et al_., 1954) while dopamine was found in the corpus striatum of different animals (Bertler and Rosengren, 1959).

UMI

With the development in the last decade of different histological and biochemical techniques based on the presence of a given type of transmitter substance or on specific synthetic enzymes involved on the formation of the transmitter, it has become possible to map chemically defined neuronal systems in the CNS. With the histochemical fluorescence method of Falck-Hillarp, it has been possible to delineate norepinephrine-, dopamine- and 5-hydroxytryptamine-containing systems in mammalian brains. (Falck and Hillarp, 1959; Falck, et al. 1962). Subsequently, a massive amount of work was done, detailing the distribution of monoamine neurons and their terminals in the CNS (Carlsson, et al., Dahlström and Fuxe, 1964; Fuxe, 1964, 1965a, b; Fuxe and Hökfelt 1966, 1969). Mechanical, electrical and chemical lesioning techniques, in combination with fluorescence histochemical methods have helped to describe the various central monoamine pathways (Carlsson, et al., 1963; Dahlström, et al., 1966; Björklund, et al., 1975; Swanson and Hartman, 1975; Andén, et al., 1966a, b; Ungerstedt, 1971a,b). More recently the development of techniques such as the microdissection method of Palkovits (Palkovits, 1973) and the sensitive radioenzymatic microassays for measuring different neurotransmitters (Saavedra, 1976) have allowed investigators to map in more detail the main areas containing these transmitters. However, the anatomical organization of monoamine pathways still represents an area of extensive research today.

B. Distribution of Central Catecholaminergic Neurons

1. Dopamine pathways

The cell bodies of the dopaminergic neuronal tracts are found primarily in four locations. One is the substantia nigra and ventral tegmental area of the mid-brain (Dahlström and Fuxe, 1964), with axonal projections ascending in the medial forebrain bundle (MFB) to the corpus striatum - the nigro-striatal dopamine system - and to the central portion of the caudate nucleus (Ungerstedt, 1971a). Another location of dopamine cell bodies is the area dorsal to the interpeduncular nucleus in the mid-brain, whose axons ascend with those of the nigro-striatal dopamine system and innervate the stria terminalis, nucleus accumbens, and olfactory tubercles - the meso-limbic dopamine system (Ungerstedt, 1971a).

The third location is the periventricular zone of the hypothalamus and the caudal thalamus (Fuxe, 1964; Björklund and Nobin 1973; Fuxe and Hökfelt, 1966). Björklund and Nobin (1973) have divided these cells into four groups: (1) the rostral periventricular cells, (2) the arcuate nucleus cell group, (3) the dorsal and caudal hypothalamic cells, and (4) the caudal thalamic cell group. The cells in the rostral and middle hypothalamus appear to be exclusively dopaminergic, while those of the caudal group include norepinephrine cells as well. The arcuate nucleus cell group is the largest one and sends axonal projections to the median eminence, to the pons intermedia, and to the neural lobe of the hypophysis (Björklund, et al., 1973). This is the so-called tuderoinfundibular dopamine system (Fuxe 1964; Fuxe and Hökfelt, 1966; Jonsson et al., 1972). Björklund et al., 1975, have evidence for the existence of an additional dopamine pathway in the rat the so called incerto-hypothalamic dopamine neurone system. On the basis of the fiber distribution of this system, a caudal and rostral part can be discriminated: the caudal part extends from the area of the dopamine-containing cell bodies in the caudal thalamus, the posterior hypothalamic area and the medial zona incerta (the A_{11} and A_{13} cell groups) into the dorsal part of the dorsomedial nucleus and the dorsal and anterior hypothalamic areas; the rostral part extends from the area of the rostral periventricular dopaminergic cell system (the A_{14} cell group) into the medial preoptic area and the periventricular and suprachiasmatic preoptic nuclei. The system probably extends into the most caudal portion of the lateral septal nucleus.

2. Norepinephrine pathways

The cell bodies of the norepinephrine-containing neurons occur in clusters within the lower brain stem, extending from the caudal medulla oblongata to the rostral pons (Dahlström and Fuxe, 1964; Ungerstedt, 1971a). Scattered noradrenergic cells also have been found in the caudal diencephalon, but their projections are unclear at present (Björklund and Nobin, 1973). Axons from some of the norepinephrine-containing cells in the medulla oblongata descend in the lateral sympathetic columns of the spinal cord and terminate at various levels (Ungerstedt, 1971a). Two major noradrenergic pathways ascend from the brain stem cell groups: a dorsal and a ventral pathway (Dahlström, et al., 1966, Fuxe, 1965a; Ungerstedt, 1971a). Axons of the dorsal pathway orginate from cell bodies in the locus coeruleus and are responsible for innervating the amygdala, hippocampus, thalamus and cerebellar and cerebral cortex (Ungerstedt, 1971a). Hartman et al. (1972) using an immunofluorescense technique utilizing dopamine b-hydroxylase as a marker, have described three major branches of the dorsal ascending pathway. One branch diverges ventrally to join the MFB; another projects dorsorostrally, below the habenula, to the midline thalamic nuclei; the third branch difuses throughout the midbrain and thalamus, terminating in the cortex, the anterior thalamic nuclei and the geniculate bodies. It is not clear at present if the dorsal branches send projections to hypothalamic nuclei. Lindvall and Björklund (1974), using a glyoxylic acid fluorescence technique for catecholamines, did not find any significant termination of locus coeruleus fibers in the hypothalamus. The locus coeruleus also gives rise to descending fibers terminating in the cerebellum and the lower brain stem (Segal, et al., 1973; Ungerstedt, 1971a).

The axons of the ventral noradrenergic pathway originating from brain stem nuclei converge with the dopamine fibers in the midbrain and ascend via the MFB to innervate the hypothalamus, the preoptic area, the septum, the thalamus and the subthalamus

UMI

(Fuxe, 1965a, Lindvall and Björklund, 1974; Ungerstedt, 1971a). Olson and Fuxe (1972) have shown that there are two components of this ventral ascending pathway: a "subcoeruleus" component and a medulla oblongata component. The "subcoeruleus" area includes the cell groups A6 and A7 (classification of Dahlström and Fuxe, 1964), as well as norepinephrine-containing cells that connect these groups. Projections from this area primarily innervate the periventricular zone of the hypothalamus and the preoptic area. The medulla oblongata component innervates the basolateral hypothalamus and the preoptic area. The cells of this component originate from the groups A_1 and A_2 (Dahlström and Fuxe, 1964) and their projections join with fibers from cell group A_5 and ascend together with the "subcoeruleus" component. Björklund et al. (1973) using various lesions of the ascending ventral noradrenergic pathway, have demonstrated that its fibers entering the rostral hypothalamus terminate bilaterally in the internal and subependymal layers of the median eminence (ME). Cuello et al. (1973b) using biochemical studies found high concentrations of NE in the ME.

There is no projection of the ventral ascending pathway to the external layer of the ME according to the findings of Björklund et al., 1973 and Jonsson et al., 1972. In contrast, Cuello et al., 1972; 1973a, found biochemical and untrastructural signes of axonal damage in the external layer of the median eminence after intraperitoneal injection of 6-hydroxydopamine and after parallel knife cuts were made along the lateral borders of the medial basal hypothalamus.

3. Epinephrine

The presence of epinephrine in the brain was first confirmed by biochemical and fluorometric determinations (Vogt, 1954; Gunne 1962) but the relative amounts were low. Recent studies using immunofluorescence histochemistry by Hökfelt et al. (1973; 1974) have shown the existence of phenylethanolamine-N-methyltransferase (PNMT) positive cell bodies and nerve terminals in the CNS of the rat. Two groups of cells were found in the rostral medulla. The first and largest, C1, was located in the ventrolateral reticular formation; and the second, C2, surrounded the medial region of the floor of the fourth ventricle. The highest concentrations of PNMT positive nerve terminals were found in various nuclei of the brain stem, locus coeruleus, periventricular gray areas of the diencephalon and mesencephalon and a few in the dorsomedial nucleus of the hypothalamus. These investigators were careful to point out that their data gives only indirect evidence that epinephrine neurons are present in the central nervous system. Further investigation must be done for mapping these adrenergic pathways.

C. Distribution of Central Serotonergic Neurons

The cell bodies of the serotonin-containing neurons are localized in a series of nuclei in the lower midbrain and upper pons that are called the raphe nuclei. Serotoninergic axons ascend primarily via the medial forebrain bundle and terminate in areas of the septum, amygdala, stiatum, neocortex and the suprachiasmatic region of the hypothalamus (Heller and Moore, 1965; Aghajanian et al., 1969; Baumgarten, et al., 1972; Palkovits, et al., 1974; Saavedra, et al., 1974, 1975).

The majority of axons leaving the suprachiasmatic nucleus pass dorsally and caudally through the periventricular area, where they are close to the periventricular nucleus neurons and to dentrites of VMN (ventromedial nucleus), DMN (dorsomedial nucleus) and ARC (arcuate nucleus). A small group of fibers appear to pass directly to the internal layer of the median eminence (Swanson and Cowan, 1975).

Axons from the caudal cell groups descend to the lower brain stem and the gray matter of the spinal cord (Fuxe, 1965a).

D. Monoamine metabolism

Brain monoamines represent molecules of norepinephrine, dopamine or serotonin that are synthesized and stored within brain neurons and are potentially releasable from these neurons as their neurotransmitter. Although most investigators would probably agree that it is likely that monoamines function as central neurotransmitters, few would claim that this point has been proven beyond all doubt.

It is generally agreed that the following operational criteria should be satisfied in order to prove that a particular compound functions as the neurotransmitter at a given synapse: (1) the compound and the enzymes necessary for its synthesis should be present in the terminal boutons of the presynaptic neuron.

UMI

Moreover, catabolic enzymes or other biochemical mechanisms for terminating the physiological activity of the putative neurotransmitters (e.g. uptake "pumps") should also be demonstrable in the region of the synapse. (2) The compound should be released from the presynaptic ending when this neuron is stimulated (i.e. while the presynaptic neuron is transmitting its signal to the postsynaptic cell). (3) Application of the compound to the postsynaptic neuron should produce physiological effects that mimic those that follow stimulation of the presynaptic neuron. (4) Drugs that act postsynaptically should block the effects of the test compound when it is applied to the postsynaptic neuron (Antontay and Wurtman, 1971).

1. Catecholamine metabolism

Catecholamine metabolism in adrenergic neurons involves the following major events: enzymatic synthesis, storage, release, receptor interaction, reuptake, and inactivation of the transmitter (Glowinski, 1970).

a. Synthesis

The first biochemical transformation in the synthesis of brain catecholamines involves the meta-hydroxylation of tyrosine which is actively taken up by adrenergic neurons and takes place throughout the neuron. However, the greatest accumulation of amine occurs at the terminal boutons as a result of axoplasmic transport, terminal synthesis and reuptake. The hydroxylation of tyrosine and formation of dihydroxyphenylalanine (DOPA) is catalyzed

UMI

by the enzyme L-tyrosine hydroxylase (TH), the rate-limiting enzyme. This reaction can be blocked by the competitive inhibitor a-methyltyrosine. DOPA is converted to dopamine (DA) by a non-specific decarboxylating enzyme, L-amino acid decarboxylase. Dopamine then enters a storage vesicle and in noradrenergic neurons, is b-hydroxylated to norepinephrine by the enzyme dopamine-β-hydroxylase (DBH). This reaction can be blocked by a dithiocarbamate derivative, FLA-63.

This enzyme appears to be present in soluble form and in particulate form within the vesicle (Axelrod, 1972). The uptake into these vesicles is an energy dependent process requiring adenosine triphosphate (ATP) and magnesium and can be blocked by reserpine and ouabain (Iversen, 1970; Von Euler and Lishajko, 1963). Although the intraneuronal uptake and conversion of dopamine represents an important step in norepinephrine synthesis, the majority of evidence favors tyrosine hydroxylase as being the regulatory enzyme in this pathway. Tyrosine hydroxylase occurs in free and membrane-bound form, the latter form being predominant in areas rich in catecholamine nerve endings (Kuczenski and Mandell 1972). Although it is not clear which form of this enzyme is regulatory in catecholamine synthesis, it is conceivable that a change in the localization of free to bound form could modify its activity (Mandell, *et al.*, 1972). In addition, the enzymatic activity of soluble brain TH has been shown to increase after limited depolymerization with trypsin (Kuczenski, 1973). This suggests that the soluble form of the enzyme may be relatively inactive until it undergoes a conformational change associated with membrane binding.

Tyrosine hydroxylase and DBH activity are affected by end-product feed-back (Axelrod, 1972; Costa, 1970; Pletscher, 1972). However, the accumulation or depletion of end-product is dependent, in part, on the degree of neuronal activity. Carlsson _et al_. (1972) have proposed that a neuronal feed-back loop regulates catecholamine synthesis. According to this view of trans-synaptic control, the postsynaptic neuron communicates with the presynaptic neuron via collaterals or interneurons, to increase or decrease its firing rate. Alternatively, the presynaptic neuron itself may have inhibitory receptor(s) at its terminals which are activated by a certain level of transmitter released in the synaptic cleft (Langer, 1973; Starke and Altmann, 1973). In conclusion, catecholamine synthesis is regulated by a very complex system of events, rather than by one single step.

The final step in CA synthesis involves the methylation of norepinephrine to epinephrine, a reaction catalyzed by phenylethanolamine-N-methyl-transferase (PNMT) (Axelrod, 1962; Hökfelt, _et al_., 1973).

b. Storage

The central and peripheral adrenergic nerve endings contain large and small (80-100 nm and 45-50 nm diameter, respectively) vesicles. These vesicles appear to have some general properties: 1) they possess an outer limiting membrane; 2) with appropriate fixation, they possess one electron dense core when viewed under the electron microscope; 3) some of those contain the enzyme-β-hydroxylase, and a soluble protein chromogranin which has been suggested to be involved in the storage process; 4) they have a high concentration of catecholamine and ATP in a 4:1 ratio; and 5) they contain Mg^{++} and Ca^{++} -dependent ATPase. A number of possible functions have been proposed for the catecholamine storage vesicles: 1) they store CA and protect catecholamines from degradation by monamine oxidase (MAO) which is considered to be intraneuronal; 2) they store different pools of transmitter which may be released upon the appropriate physiological stimulus; 3) they oxidize dopamine to norepinephrine; 4) they take up dopamine from the cytoplasm. Dopamine seems to be stored in the small dense vesicles, while norepinephrine is stored by both types of vesicles (Fuxe and Hökfelt, 1969; Bloom and Aghajanian, 1968). However, small quantities of amine are also present in a very loosely bound form outside the vesicles. The mechanisms by which the extravesicular amine is protected against inactivation is still unknown (Glowinski, 1970).

Sucrose gradient centrifugation studies have shown two types of noradrenergic storage vesicles; one sediments as a "heavy" fraction and contains high levels of DBH and chromogranin. The other sediments as a "light" fraction and contains very little DBH activity (Roth, et al., 1968; Chubb et al., 1970). Subsequently, it was shown that the heavy fraction containing large vesicles and the light fraction, mainly small granular vesicles (Bisby and Fillenz, 1971). These authors report that most terminal stores of norepinephrine are of the light fraction type. Besides this morphological information, the concept of a heterogeneous distribution of catecholamine in neurons or even in nerve endings has been developed on the basis of pharmacological and biochemical data (Glowinski and Iversen, 1966; Kopin et al., 1968; Huges and Roth, 1974). It appears that NE as well as DA are localized in pools or compartments which are different not only between NE and DA systems of the CNS but also between various systems of NE or DA neurons.

Dahlström and Häggendall (1972) have put together the morphological information and the concept of the transmitter pools to derive a hypothetical model. According to them, the large dense core vesicles seen in electron micrographs, and sedimenting as a heavy fraction on sucrose gradients, represent the new amine granules. These are formed in the cell bodies, and are transported down the axon to the terminals, becoming part of a small easily releasable or functional pool of transmitter. The small dense core vesicles, which sediment as light particles, constitute a large, more stable pool of old amine granules which may act as storage reservoirs.

The storage of catecholamines can be blocked by Rauwalfia alkaloids which interfere with the storage of monoamines by blocking the uptake of amine into the storage vesicles or disrupting the binding of the amines with the protein.

c. Release

Much of the current knowledge regarding the release of cathecholamines derives from the study of both adrenal medullary tissue and adrenergically innervated peripheral organs. Most approaches to the study of CA release in CNS have relied upon the initial labeling of endogenous amine stores, followed by determination of labeled amine output under conditions of electrical, ionic or drug-induced stimulation (Farnebo, 1971; Besson *et al.*, 1971; Thierry *et al*, 1971). These studies support the idea that amines are released from storage vesicles into the synaptic cleft or extracellular space, probably by a process of exocytosis, requiring the presence of extracellular calcium (Farnebo, 1971). And, unlike the processes of uptake and storage, release does not appear to be an energy-dependent process (Farnebo, 1971).

d. Receptor interaction - postsynaptic action of catecholamines

It is generally accepted that the initial step in a chain of events is an interaction between the neurotransmitter and the specific receptor of the postsynaptic neuronal membrane. The following events are postulated to occur:

Neuronal stimulation causes release of a transmitter which then impinges on the postsynaptic cell. Here the transmitter activates adenylate cyclase to increase the rate of production of the cyclic nucleotide, adenosine -3', 5'-cyclic monophosphate (CAMP). This nucleotide activates a protein kinase to phosphory-

UMI

late, via ATP either some protein substrate, which could then alter the resistive and capacitative properties of the postsynaptic membrane, or a nuclear histone and affect the metabolic properties of the postsynaptic neuron. The finding of adenyl cyclase in the CNS by De Robertis, _et al_., 1967, and the similar measurement of CAMP-dependent protein kinase by Maeno, _et al_., 1971, supports this hypothesis. In addition to CAMP, prostaglandins have also been implicated in the postsynaptic effects of neurotransmitters (Hedquist, 1973; Hoffer, _et al_., 1972). Recent evidence has indicated that pre-synaptic receptors also exist and bear identical characteristics to the postsynaptic receptors for their respective transmitter. They may be involved in the neuronal feed-back loop regulating catecholamine biosynthesis (Langer, 1973).

e. _Reuptake - inactivation_

Two major mechanisms account for the inactivation of released catecholamine: enzymatic degradation and reuptake. Enzymatic catabolism occurs by two pathways: oxidative deamination by monoamine oxidase (MAO) and 3-o-methylation by catechol-o-methyltransferase (COMT). Monoamine oxidase is an enzyme localized largely in the outer membrane of mitochondria (Cotzias and Dole 1951), which is responsible for the intraneuronal degradation of catecholamines and converts these substances to their corresponding aldehydes. Monoamine oxidase is usually considered to be an intraneuronal enzyme, but it also occurs in abundance extraneuronally. The catechol-o-methyltransferase enzyme has an extra-neuronal location, is a relatively non-specific enzyme and catalyzes the transfer of methyl groups from s-adenosyl methionine in the presence of Mg^{++} ions to the m-hydroxyl group of catecholamines and various other catechol coumpounds. However, the primary mechanism for inactivating released amines appears to be the reuptake into the presynaptic terminals (Andén, *et al.*, 1969) where it may then either be metabolized by MAO or recaptured by one of the transmitter pools (Iversen and Glowinski, 1966).

2. Serotonin (5-HT) metabolism

The first step in serotonin synthesis appears to be the uptake into the neuronal cell of the amino acid L-tryptophan, the primary substrate for this neurotransmitter. Plasma tryptophan arises primarily from the diet, and elimination of dietary tryptophan can profoundly lower the levels of brain serotonin. The uptake is an active process and the entry site into the cell can be competed for by certain other amino acids, such as phenylalanine (Anton-Tay,1971). The next step in the pathway of serotonin synthesis is the hydroxylation at the 5 position to form 5-hydroxytryptophan (5-HTP). The enzyme responsible for this reaction is tryptophan hydroxylase. It is found in the brain (Grahame-Smith 1964; Snyder and Axelrod, 1966) and appears to have an absolute requirement for molecular oxygen and reduced pteridine cofactor, and also requires a sulfhydryl stabilizing substance, such as mercaptoethanol for preservation of activity at least in vitro. Tryptophan hydroxylase appears to be a soluble cytoplasmic enzyme. However, investigators examining the relative distribution of particulate and soluble tryptophan hydroxylase have reported that the particulate form of the enzyme may be associated with 5-HT-containing synapses, while the soluble form is more likely to be associated with the cell body cytoplasm (Wurtman, 1970). This step in the synthesis can be blocked by a synthetic amino acid p-chlorophenylalanine, which competes directly with the tryptophan and also binds irreversibly to the enzyme (Koe and Weissman, 1966). Following its synthesis, 5-HTP is almost immediately decarboxylated to yield serotonin (5-HT). The enzyme responsible for this reaction is the non-specific aromatic L-amino acid decarboxylase enzyme found in the brain (Grahame-Smith, 1964; Snyder and Axelrod, 1964), and mainly associated with the synaptosomal fraction. Since the decarboxylation reaction occurs very rapidly and since its Km requires less substrate than the preceding step, tryptophan hydroxylase would appear to be the rate-limiting step in the synthesis of 5-HT. When this enzyme is inhibited, the serotonin content of the brain rapidly decreases. On the other hand, when the 5-HTP decarboxylase is inhibited by equal or greater amounts there is no effect upon the level of brain 5-HT. Serotonin also appears to be stored in vesicles within nerve endings. After the appropriate stimulus, it is assumed that serotonin is released from the presynaptic cell into the synaptic cleft and affects the postsynaptic membrane specific receptor, to initiate a cascade of biochemical events in the postynaptic cell. Serotonin then is retaken up in the presynaptic neuron where it is deaminated by the enzyme monamine oxidase. The product of this reaction, 5-hydroxyindoleacetaldehyde can be further oxidized to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol depending on the NAD^+/NADH ratio in the tissue (Snyder, 1972).

Although there is a relatively brief sequence of synthetic and degradation steps involved in serotonin metabolism, there is still much to be learned regarding the physiological mechanisms for controlling this pathway. Several studies suggested that serotonin synthesis is "turned off" by intraneuronal 5-HT, by means of end-product inhibition. Others have proposed exactly the opposite and suggest that 5-HT synthesis is independent of the intraneuronal amine levels (Hamon et al., 1973). Recent evidence based on pharmacological studies (Johnson, et al., 1972; Hidaka, 1971) and lesion studies (Samanin and Garattini, 1975) suggests a functional link between adrenergic and serotonergic neurons in the brain and it may well be that serotonin synthesis and/or metabolism are under a regulatory influence of catecholamines in the brain.

E. Experimental Approaches to Study Brain Amine Function

There are several methods which are used to affect brain amine levels. In general these include (1) treatment with drugs which affect the different steps in the metobolism of the amine, (2) mechanical disruption of monoamine pathways by lesions or cutting the fibers, (3) chemical destruction of monamine-containing neurons with selective drugs and (4) iontophoretic application of drugs or transmitters in various regions of the brain. Each of these methods used alone or in combination although having limitations, complexities and secondary effects, have helped to develop our understanding about the CNS control of different endocrine functions.

III. Monoamine Involvement on Luteinizing Hormone (LH) Release

Early evidence for participation of catecholamines in the regulation of gonadotropin secretion was obtained by Sawyer and co-workers (Sawyer, et al., 1947) who demonstrated that ovulation can be blocked by dibenamine. Sawyer and his associates had also shown that drugs such as reserpine which deplete monoamines can block ovulation and lead to the development of pseudopregnancy in animals (Barraclough and Sawyer, 1959). If the depletion of monoamines is prevented by the administration of monoamine oxidase inhibitors such as pargyline, or precursors of catecholamines, the effects of reserpine are reversed which indicates that they are due to the depletion of amines (Coppola, et al., 1965; Coppola, 1968).

Additional evidence supporting a role for catecholamines in the regulation of gonadotropin secretion has been provided by the demonstration of alterations in hypothalamic amine content and turnover, monoamine oxidase activity, and other enzymes in the whole brain or in hypothalamus or even in isolated nuclei in the hypothalamus (Donoso et al., 1967; Anton-Tay and Wurtman, 1971; Kato and Minaguchi, 1964; Zolovick et al., 1966; Kamberi and Kobayashi, 1970; Kizer et al., 1974; Selmanoff et al., 1976) with the stage of estrous cycle or following alteration in the blood level of gonadal steroids.

Supporting evidence is the finding of Fuxe and Hökfelt (1969) using fluorescense microscopy. They have shown the presence of catecholaminergic and serotonergic neurons in the hypothalamus. A role for these particular neurons in the control of gonadotropin secretion is suggested by the alterations in their content of dopamine in situations associated with altered gonadotropin secretion.

Further studies, using in vitro methods and in vivo techniques such as intraventricular administration of monamines or by administration of drugs affecting different biosynthetic steps of monoamines, support the idea that noradrenergic neurons play a role in stimulating the release of LH (Sawyer _et al_., 1947; Kalra, _et al_., 1972; Ojeda and McCann, 1973; Kalra and McCann, 1973; Sawyer _et al_., 1974; Cocchi _et al_., 1974). However, dopamine has been implicated in both excitatory and inhibitory influences in the regulation of LH secretion (Kamberi, _et al_., 1969; Ahren, _et al_., 1971; Fuxe, _et al_., 1972; Miyachi, _et al_., 1973).

Serotonin-containing neurons have been implicated in the inhibition of LH secretion (Kordon, _et al_., 1968; Kordon, 1971; Kamberi, _et al_., 1970; Labhsetwar, 1971; Brown, 1967).

However, recent development of highly sensitive enzymatic microassays for measuring monoamines, the microdisection technique and other biochemical approaches, provide help for further investigation in the answering of many questions concerning the actual mechanism of monamine involvement in the regulation of LH release.

STATEMENT OF PURPOSE

Central catecholaminergic and serotonergic neurons have been implicated in the control of gonadotropin secretion by a number of investigators. Although neurotransmitters, e.g. certain biogenic amines, are believed to act as modulators linking neural stimuli and the cells which produce gonadotropin releasing factors, the precise role of such transmitters is equivocal. Norepinephrine has been suggested to play an excitatory role in the neural events controlling the release of luteinizing hormone (LH) (Sawyer, *et al*., 1947; Sawyer, *et al*., 1949; Kalra, *et al*., 1972; Ojeda and McCann 1973; Kalra and McCann 1973; Sawyer, *et al*., 1974; Cocchi, *et al*., 1974). The role if any of dopamine is not as clear since this putative neurotransmitter has been implicated in both excitatory (Kamberi, *et al*., 1969; Schneider and McCann 1969; Kordon and Glowinski 1969; Schneider and McCann 1970; Choudhury, *et al*., 1974), and inhibitory influences in the regulation of LH secretion (Ahren, *et al*., 1971; Uemura and Kobayashi 1971; Miyachi, *et al*., 1973).

Serotonin is thought by some to inhibit gonadotropin release (Kordon, et al., 1968; Fraschini, et al., 1971; Kordon 1971; Kamberi, et al., 1970; Labhsetwar 1971). However, Brown (1967) believes that serotonin stimulates gonadotropin release under some conditions.

The confusion and discrepancies which revolve around neurotransmitters as modulators of LH-RH neurons may be due in part to the use of different experimental conditions and animal models. For example, it is known that modification of an animal's hormonal state can alter the hypothalamic composition of endogenous catecholamines and indoleamines(Lichensteiger, et al., 1960; Kato, 1960; Donoso and Stefano, 1967; Donoso, et al., 1967, 1969; Fuxe, et al., 1969, 1971) Furthermore, it is recognized that the efficacy of exogenously administered transmitters may be influenced by certain barriers (e.g. blood-brain barrier, or monoamines placed in a ventricle may not penetrate more than 300 μ into the periventricular tissue (Fuxe and Ungerstedt, 1968), which may prevent the transmitter from reaching its site of action. In addition, pharmacological manipulations of the life-cycle of the neurotransmitter are not, in many cases, specific for only one transmitter and changes in the levels of one transmitter may also be reflected in changes in other neurotransmitter levels.

Ovariectomized rats used throughout this project have a demonstrable episodic LH release pattern and high blood levels of LH, measurable by the ovine-ovine rat LH double antibody radioimmunoassay method for whole blood (Osland, et al., 1975). Thus, they serve as a model to examine possible neurotransmitter involvement in LH-RH release preceding the episodic discharge of LH. Various studies have suggested that the brain may play a significant role in the origin of these episodic LH bursts, and that the pituitary is not the site of origin of the mechanism underlying episodic LH release (Bremner and Paulsen, 1974; Osland, et al., 1975; Carmel, et al., 1975; Bledsoe and Ramirez, 1976). Few studies have explored brain catecholamine involvement in the episodic release of LH. Although Santen and Bardin (1973) failed to show any effect of a-adrenergic blocking agents on pulsatile LH release in men, Bhattacharya, et al. (1972) have reported that a-adrenergic blocking agents (phenoxybenzamine and phentolamine) and neuroleptic agents (haloperidol and chlorpromazine) blocked episodic LH release in the ovariectomized monkey. Gnodde and Schuiling (1976) have reported that administration of phenoxybenzamine inhibited episodic LH release in ovariectomized rats. Beta-adrenergic blockade, in contrast, was without effect on pulsatile LH release in monkeys (Bhattacharya, et al., 1973). The effectiveness of a-adrenergic receptor blockade in inhibiting episodic LH release suggests catecholamine involvement in pulsatile LH discharge. Episodic LH release persists following complete deafferentation of the medial basal hypothalamus in ovariectomized monkeys (Krey, et al., 1975) and rats (Blake and

Sawyer 1974). However, the norepinephrine content of the deafferented hypothalamus is depleted by this procedure (Weiner, et al., 1972). This would suggest that norepinephrine may not play a role in episodic LH release. Moreover, recent studies (Gallo 1976) suggest a possible involvement of serotonin in the inhibitory effect on episodic LH release produced by stimulation of the arcuate nucleus in the hypothalamus. Other studies have shown that treatment with estrogen can eliminate the pulsatile LH rhythm in ovariectomized monkeys (Knobil 1974) and ovariectomized rats (Blake, et al., 1974).

The present experiments, therefore, are aimed at clarifying the possible role of brain norepinephrine, dopamine and serotonin in episodic release of LH in ovariectomized rats.

MATERIALS AND METHODS

Animals

Adult female Sprague Dawley rats (Simonsen Laboratories, Gilroy, California) were maintained on a 14 hour light : 10 hour dark schedule (lights on 5:00 AM to 7:00 PM) and fed Feedstuffs Processing rat food (San Francisco, California) and water *ad libitum*. Daily vaginal smears were taken and rats showing two or more consecutive 4-day estrous cycles were ovariectomized. Rats weighing 290-360 g were tested three to eight weeks after ovariectomy.

Bleeding Procedure

On the day prior to the experiment, a polyethylene cannula was inserted through the right external jugular vein to approach or enter the right atrium. Rats were acclimated for several hours to the laboratory space in which the experiments would take place, and were then returned to the animal quarters overnight. The following day, the cannula was connected to one end of a piece of flexible tubing (PE 50, Technicon Corporation, Tarrytown, New York) which was inserted into a peristaltic pump (LKB, Los Angeles, California). The other end of this tubing was connected to a microliter syringe kept on ice for the collection of blood samples. Bleeding was generally done between

10:00 AM and 6:00 PM. Two hundred units of heparin were injected intravenously 30 minutes prior to the onset of bleeding, and a smaller supplemental dose given if needed. Unanesthetized, unrestrained rats were bled continuously at a rate, depending on the experiment, of either 30, 50 or 100 µl whole blood every 4-6 minutes for several hours. In cases where drugs were administered during the bleeding period a 1½ to 2 hour period of bleeding preceded this treatment to determine the episodic LH release pattern in that animal. Blood was added directly to assay tubes containing an appropriate amount of phosphate buffered saline (PBS) with 0.1% gelatin. Assay tubes were kept in an ice bath. Hematocrits remained stable during the course of several hours of bleeding (onset 43.4, end 41.9; n = 36).

Radioimmunoassay of LH

A. General principles of the assay

The radioimmunoassay (RIA) is based on the following immunological reaction:

antigen (Ag) + antibody (Ab) → Ag-Ab complex.
Hormone + antihormone → hormone-antihormone complex.

Antigens are substances which can react specifically with antibodies and under appropriate conditions can induce an animal to form specific antibodies. An antibody is a substance which can be induced in an animal by an antigen and which reacts specifically with the antigen.

UMI

Specifically, the RIA involves a competition between an unlabeled hormone and a highly purified radioactively labeled hormone for limited antibody sites. The labeled and unlabeled hormones are bound to the antibody in a ratio which is proportional to their concentrations. This provides a combination of the sensitivity of radioisotope methods with the specificity of immunochemical procedures.

In radioimmunoassay, it is assumed that: a) radioactively labeled and unlabeled hormones behave identically; b) the circulating hormone is chemically and immunologically identical with the standard hormone preparation; c) the amount of hormone bound by the antibody is dependent on the concentration of the hormone; d) different antisera made against a particular antigen give similar quantitative responses in the immunoassay system; and e) there is no significant isotope effect.

B. Steps

The ovine-ovine rat LH double antibody radioimmunoassay method of Niswender *et al* (1968) was employed, except that PBS with 0.1% gelatin was used as the diluent. The basic steps involved in the assay are:

Day 1: a) Addition of purified ovine LH (used in different amounts to obtain a standard curve) and whole blood containing unknown amounts of hormone being assayed. b) Addition of antiserum, or specific antibody, prepared by injecting purified antigen (e.g. ovine LH) into another animal (rabbit, goat, etc.) for production of relatively specific antibodies against the ovine LH.

Day 2: Addition of highly purified antigen (e.g. ovine LH) reacted with radioactive iodine (^{125}I) to produce a labeled antigen (e.g. iodinated ovine LH).

Day 3: Addition of a nonspecific antibody prepared by injecting gamma globulin protein from the species in which the first antibody is developed into a second system to produce antibodies against gamma globulin. Since antigen-antibody complexes are soluble at the high dilutions used in RIA, a separate step must be used to separate antibody-bound hormone from free hormone. The double antibody method is utilized generally for the separation. The second antibody causes precipitation of the complexes while the free unbound hormone remains in solution.

Day 4-6: Incubation of assay tubes under refrigeration.

Day 7: Pour off unbound material and count radioactive precipitate.

The anti-ovine LH serum was provided courtesy of Dr. G. D. Niswender and diluted 1:40,000. The highly purified ovine LH for radioiodination (^{125}I) was a generous gift from Dr. H. Papkoff. The second antibody (ovine antiserum against rabbit gammablobulin) was kindly provided by Dr. E. F. Ellington and diluted 1:60. Rat LH, used as the standard for the assay, was provided by NIAMDD. LH values (ng/ml whole blood) were expressed in terms of the NIAMDD Rat LH-RP-1 reference preparation, which has a biological potency equivalent to 0.03X NIH-LH-S1.

C. Sensitivity

Analysis of multiple replicate samples of rat serum pools measured at high, intermediate and low ranges of the standard curve revealed coefficients of variation ranging from 8-18% for interassay variability, and 5-15% for intraassay variability.

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Pharmacologic Agents

A. Drugs affecting catetholamine or serotonin biosynthesis or acting on adrenergic or dopamine receptors were tested as follows: DL-a-methyl-p-tyrosine methyl ester hydrochloride (αMPT, a tyrosine hydroxylase inhibitor {Spector, et al, 1965}, Aldrich Chemical, Milwaukee, Wisconsin, 250 mg/kg ip in saline, 3 hours prior to experiment); 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624, a dopamine-β-hydroxylase inhibitor {Johnson, et al, 1970}, Aldrich Chemical, Milwaukee, Wisconsin, 200 mg/kg ip in a 0.25% methylcellulose suspension 18 hours prior to the experiment); bis (4-methyl-1-homo-piperanzinyl thiocarbonyl) disulfide (FLA-63, a dopamine-β-hydroxylase inhibitor {Corrodi et al., 1970}, Aldrich Chemical, Milwaukee, Wisconsin, 25 mg/kg ip in acid saline 3 hours prior to experiments); dl-Threo β-3-4 dihydroxyphenylserine (DOPS, a precursor of norepinephrine {Creveling, et al., 1967), Regis Chemical Company, Chicago, Illinois, 250 mg/kg ip in acid saline, given during the bleeding period); isoproterenol (isuprel, a β-adrenergic receptor stimulating agent, Winthrop Labs, New York, 12 μg/kg sc); apomorphine hydrochloride (a selective stimulator of dopamine receptors {Anden, et al., 1967, Ernst 1970}, Merck Chemical, Rahway, New Jersey, 1.50 mg/kg ip or sc in saline given during the bleeding period); Piribedil methane sulfonate, 1-(2-pyrimidyl)-piperonyl-piperazine (ET495, a long acting selective stimulator of DA receptors (Corrodi, et al., 1971, Walters and Roth, 1974), Les Laboratoires Servier, Nevilly, France, 20 mg/kg ip in 0.2-0.3 cc distilled water given during the bleeding period); Pimozide (a blocker of dopamine receptors (Janssen, et al., 1968), Janssen Pharamceutical, Beerse, Belgium, 1.26 mg/kg sc in tartaric acid 16 hours and 6 hours prior to apomorphine); dextro-butaclamol-AY-23.028, a blocker of dopamine receptors (Voith and Herr, 1975, Lippmann and Pugsley, 1975), Ayerst Research Lab, Montreal, Canada, 0.5 and 1mg/kg ip in saline containing 1 drop of Tween 80, 3-4 hours prior to systemic administration of apomorphine or ET495 or 4-5 hours prior to dopamine hydrochloride infusion into III ventricle); and levo-butaclamol (an inactive form of butaclamol (Lippmann, et al., 1975), Ayerst Research Lab, Montreal, Canada, 1mg/kg ip in saline containing 1 drop of Tween 80, 3-4 hours prior to apomorphine or ET495); p-chloro-phenylalanine, a drug affecting serotonin biosynthesis (PCPA, an inhibitor of tryptophan hydroxylase (Koe and Weissman, 1966) Calbiochem, 32mg/100g bwt in saline ip 70 hours prior to apomorphine).

b. Intraventricular infusion of neurotransmitters: 24µl of artificial cerebrospinal fluid (CSF) plus 1 µl 0.1% ascorbic acid as an antioxidant, containing the agents listed below, were infused for 1-1½ hours by means of an infusion pump (Sage instruments) and Hamilton microliter syringe through a small cannula inserted into the third ventricle. The agents used were 2.5, 5, 10µg DA 4-5 hours after the administration of d-butaclamol; 0.5, 1, 2.5, 5, 10, 20µg L-Norepinephrine (NE) bitartrate, Sterling Winthrop; 10, 20, 50µg NE 18 hours after the administration of U-14,624; 5, 10, 20 µg after the administration of estradiol benzoate; 5, 10 µg Serotonin

(5HT; 5-hydroxytryptamine) creatinine sulfate complex hydrate, Calbiochem.

Estradiol benzoate in oil (5 μg/100 g bwt/day for 2 days) was given sc prior to systemic administration of apomorphine or ET495, or NE infusion into the third ventricle.

Corticosterone Measurements

In rats treated with apomorphine or ET495 alone or following dextro-butaclamol, changes in plasma corticosterone levels were measured by a competitive protein binding radioassy (Murphy, 1976). Samples of whole blood (0.4cc) were taken with a heparinized syringe at approximately 20 minute or 1 hour intervals, depending upon the experiment, during the control bleeding periods and after different treatments. Blood samples were replaced with equal volumes of saline. Whole blood was centrifuged at 4°C and the plasma stored frozen at -25°C until subsequent radioassay for corticosterone.

Measurements of Catecholamines in Hypothalamus

In individual ovariectomized rats, changes in hypothalamic levels of dopamine and norepinephrine after drug or vehicle administration were determined by a modification of the radioisotope-enzymatic catechol-o-methyltransferase assay {COMT; Coyle and Henry 1973}. All rats in this study were ovariectomized 3 weeks prior to drug treatment. The animals were treated with a-MPT, U-14,624, FLA-63 or their respective control solutions (saline for a MPT, acid saline for FLA-63, and methylcellulose for U-14,624) and sacrificed by decapitation. Their brains were rapidly removed and frozen on dry ice. Hypothalami, weighing 40 to 50 mg, were bounded rostrally by the optic chiasm caudally by the mammillary bodies, laterally by the hypothalamic sulcus (immediately medial to the temporal lobe) and dorsally by a plane parallel to the base of the brain through the ventral margin of the anterior commissure. These fragments were dissected out and assayed within 24 hours for both dopamine and norepinephrine.

A. Main steps of the COMT assay

The assay employs ^{3}H-S-adenosylmethionine (^{3}H-SAM) as a cofactor in the enzymatic conversion of norepinephrine or dopamine to a labeled methylated derivative.

1. Incubation reaction. The COMT enzyme was extracted from rat liver. The reaction includes buffered radioactive COMT enzyme solution plus substrate at pH 9.1 for 30 minutes at 37°C and leads to the formation of methoxytyramine and normetanephrine.

2. Organic extraction I. a) Add Toluene-Isoamyl alcohol (3:2), vortex, centrifuge, and discard aqueous layer. b) Extract toluene phase with 0.1 N HCl, vortex, centrifuge, and discard organic phase.

3. Periodate cleavage reaction. At 2 minute intervals add sodium metaperiodate followed by 10% glycerol to stop the reaction. This leads to the formation of methoxytryamine and vanillin.

4. Organic extraction II; Extraction of cleavage products with Toluene. Vanillin is unchanged and stays in Toluene phase. Methoxytyramine is charged and is in aqueous phase. Count aqueous phase for dopamine (^{3}H methyl Dopamine) and toluene phase for norepinephrine (^{3}H methyl vanillin).

B. Sensitivity and specificity of the COMT assay

As in the original publication of this method (Coyle and Henry 1973), metabolites of the catecholamines and other substances were checked for assay reactivity and were shown not to interfere in the results. L-Epinephrine not unexpectedly interferes, but it has not been shown to exist in significant quantities in the rat CNS (Gunne 1962, Hökfelt et al, 1973, 1974).

The assay shows a constant coefficient of variation, over the range 0-2 ng for both NE and DA. Since tissue factors inhibit the enzyme, samples were routinely run at constant weight/volume ratios (weight of tissue fragment to volume of 0.1N perchloric acid used to extract the catecholamines).

Infusion into the III Ventricle

A. Preparation of artificial CSF: The following solution of artificial CSF was made according to the method of Palaic et al 1967, kept refrigerated and used as a basic stock solution in each 2 week period of experiments: NaCl 58.45 g/liter of distilled water; KCl 2.61 g/l; $CaCl_2$ 1.4 g/l; $Mgcl_2$ $.6H_2O$ 2.35 g/l; NaH_2PO_4 $.H_2O$ 0.7 g/l; $(NH_2)_2$ Co (urea) 1.3 g/l; glucose 6.1 g/l. On the day of the experiment, it was diluted 1:10 with distilled water. To bring the dilute solution to a desired pH of ≃ 7.6, $NaHCO_3$ and NaCl were added to yield respective concentrations of 2.1003 and 1.1691 g/l. Then the pH was adjusted to 7.32 by bubbling 5% CO_2 through the solution.

DA, NE and 5HT in different doses, were dissolved just before infusion into the III ventricle, in 25 µl artificial cerebrospinal fluid containing 1 µl of 0.1% ascorbic acid. The ascorbic acid was used at this concentration to protect the neurotransmitters from degradation, (M. H. Weinberger et al., 1975) The pH of the final solution was adjusted again to 7.32 by bubbling 5% CO_2 through it if needed.

B. Cannulation of the III ventricle

Stereotaxic implantation of a 22 gauge stainless steel cannula into the third ventricle was carried out under sodium pentobarbital anesthesia (3.5 mg/100 g bwt). Each cannula was 18 mm in length and was fitted with an inner stylet (27 gauge tubing) to prevent the escape of cerebrospinal fluid from the ventricle when the cannula was in position. In addition, each cannula was fitted with an outer sleeve of 18 gauge tubing (5mm in length) which acted as a guide tube for lowering the cannula into the ventricle.

After positioning the animal in the stereotaxic instrument (Trent H. Wells, Jr. Mechanicals Developments Company, South Gate, California), a small window was made in the skull with borders approximately 2 mm anterior and posterior to the bregma and 1.5 mm lateral to the sagittal suture. Four anchor screws were placed in the skull around the window. The stereotaxic carrier was lowered until both the cannula and outer guide tube rested on the surface of the brain, directly above the sagittal sinus and at A 6.0 (co-ordinates according to DeGroot, 1959). Small pieces of gelfoam (Upjohn) were used to protect the exposed surface of the brain from the dental cement and dental acrylate, which were placed around the guide tube and the four screws to fix the tube to the skull. After the

UMI

cement had hardened, the cannula was lowered several millimeters into the brain and then held in position with small forceps while the sterotaxic carrier was raised and removed. The cannula could then be lowered manually, a millimeter at a time, until it penetrated the ventricle. In order to keep the cannula free of brain tissue, it was cleared with a piece of 27 gauge tubing of the same length. When a continuous flow of CSF was observed at the tip of the cannula, the inner stylet was inserted and cement built up around the cannula. When the animals recovered from the anesthesia, they were returned to their cages for not less than 5 days prior to the experiment day.

C. Histological localization of cannula

The accuracy of the implantation was confirmed by reflux of cerebrospinal fluid on the day of experiment and also by histological procedure. At autopsy, after the experiment, the brains were fixed in 10% formalin + 1% $CaCl_2$ and embedded in gelatin. Frozen serial sections (50 μ) were stained with a Nissl stain using carbol fuschin.

D. III ventricle infusion set up

Approximately 1 hour before the experiment, under light ether anesthesia, the inner stylet was taken out and cerebrospinal fluid flow was checked. Another inner stainless 27 gauge tubing of the same length was inserted into the ventricle through the implanted 22 gauge cannula. It had been previously connected to one end of a piece of polyethylene tubing (PE 10) approximately 40 cm in length containing the appropriate solution of infusion. A plastic tube was used for protection of the PE 10 tube and was fixed with dental cement firmly to the skull. Aluminum foil was used to cover the entire tubing

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assembly and protect the transmitters from exposure to light. When the animals were recovered from the light ether anesthesia (usually 30 minutes) the experiment started. After a 1-1½ hour control bleeding period, the appropriate solutions were delivered at a rate of 25λ/hour through the other end of the PE 10 tube by means of an infusion pump. Post infusion blood sampling was continued for 1 hour.

Detection of Ovulation

Before the "critical" period of LH release on proestrus (Everett 1964), the animals were injected with α-MPT (-3 hours), U-14,624 (-18 hours) or their control solutions. The next morning the ovaries were removed and the oviduct dissected free under a microscope and placed on a drop of water on a slide. The diluted ampulla, containing the ova, was located and the eggs counted.

Statistics

Statistical differences in brain catecholamines between drug-treated rats and controls were examined by means of the unpaired student's *t* test.

Statistical differences in plasma corticosterone were examined by means of the paired student's *t* test.

In both statistical analyses, differences with $p<0.05$ were regarded as significant.

RESULTS

Nine ovareictomized rats were bled and their episodic LH release patterns determined, as shown for 2 animals in Figure 1. Bursts of LH release occurred every 20-40 minutes, in agreement with Gay and Sheth (1972) and Blake (1974). The LH rhythms were reasonably constant throughout prolonged bleeding periods. Mean blood LH levels were markedly similar for each rat during the bleeding period which was conducted between 10 AM and 6 PM. This is in agreement with the finding of Blake (1974) that a rise in circulating LH levels does not occur during the afternoon hours in long-term ovariectomized rats.

I. Administration of drugs affecting catecholamine or serotonin biosynthesis or acting on adrenergic or dopamine receptors.

1. αMPT

Episodic LH release was not altered in 8/8 rats treated with αMPT (250 mg/kg ip). One example is presented in Figure 2. This rat was bled before αMPT treatment and then again 3 hours following administration of the drug. However, no change occurred in the pattern of episodic LH release. Similar treatment of intact rats 3 hours prior to the critical period for LH release at 2 PM on proestrus blocked ovulation in 5/6 cases. Saline injections had no effect. Two rats were given a half-dose of αMPT 29 hours prior to bleeding, and a full dose 3 hours before bleeding, yet episodic LH release was not altered.

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Figure 1. Patterns of episodic LH release in two ovariectomized rats.

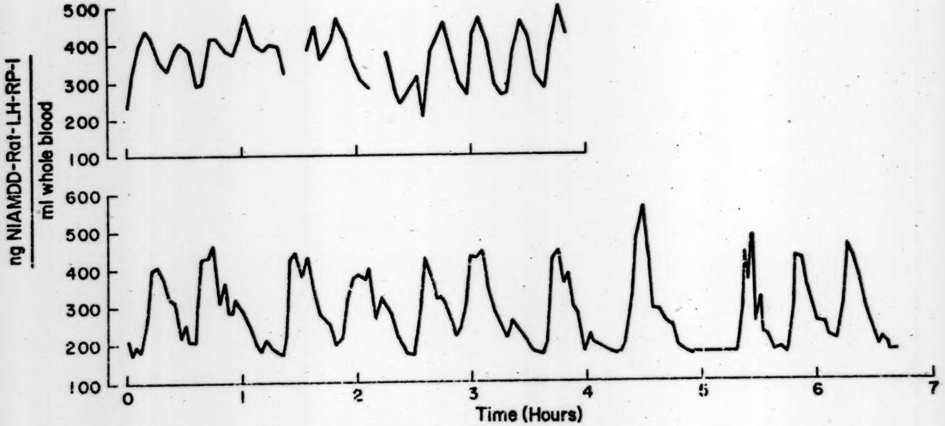
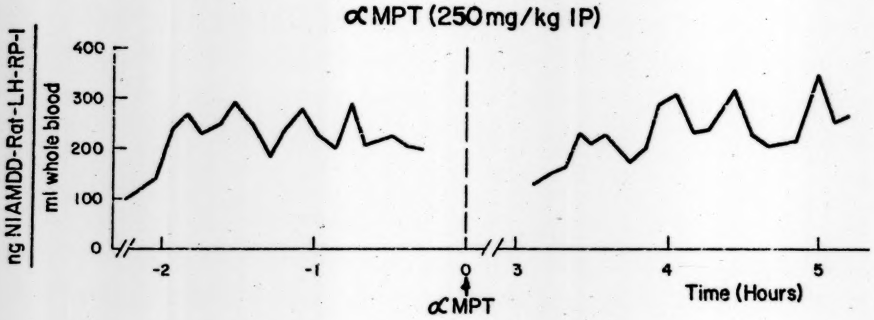


Figure 2. Effect of αMPT (250mg/kg ip) on episodic LH release in an ovx rat. This rat was bled prior to αMPT and then again three hours later. Arrow indicates time of αMPT injection.



2. U-14,624 and FLA-63

In 13/13 cases U-14,624 (200 mg/kg ip) caused a lowering of blood LH levels and blockage of episodic LH release (Figure 3). Similar treatment of intact rats 18 hours prior to the critical period for LH release at 2 PM on proestrus blocked ovulation in 6/6 rats. Control injections of methylcellulose had no effect. FLA-63 decreased blood LH levels and greatly reduced if not eliminated episodic LH release in both rats tested (Figure 3).

3. Hypothalamic catecholamines

To relate changes in episodic LH release to alterations in hypothalamic catecholamine levels, changes in the levels of dopamine and norepinephrine in individual rats were determined following treatment with αMPT, U-14,624, FLA-63 or their control solutions. By 3 hours following αMPT (Figure 4), levels of norepinephrine had decreased significantly ($P<0.001$) from 1.75 μg/g to 0.92 μg/g of hypothalamic tissue. Dopamine concentration also decreased significantly ($P<0.001$) from 0.61 to 0.25 μg/g of hypothalamic tissue. Therefore, although αMPT decreased the levels of both hypothalamic catecholamines, it failed to alter episodic LH release.

U-14,624 caused a 90% reduction in hypothalamic norepinephrine levels (2.23 to 0.19 μg/g tissue, $P<0.001$) 18 hours following its administration (Figure 5). A small but not statistically significant increase ($P>0.05$) in hypothalamic dopamine levels occurred. A similar effect was seen 3 hours following FLA-63 (Figure 5). Again, a marked reduction (80%, $P<0.001$) in hypothalamic norepinephrine from 2.10 to 0.40 μg/g of tissue and a small but not statistically significant increase ($P>0.05$) in hypothalamic dopamine resulted. Therefore, both of these

Figure 3. Effects of U-14,624 and FLA-63 on episodic LH release in individual ovx rats.

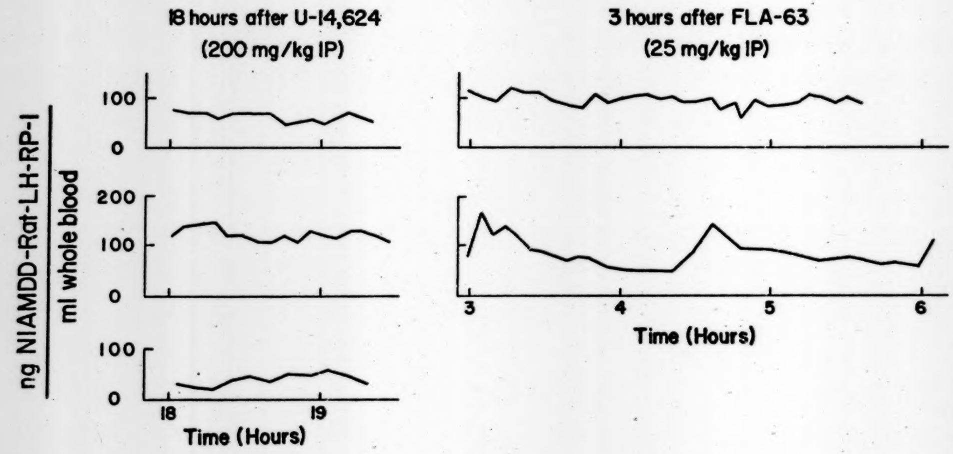


Figure 4. Effect of saline (stippled bars) or αMPT (250mg/kg ip; open bars) given three hours earlier on hypothalamic levels of norepinephrine and dopamine in individual ovx rats. Values given represent mean + SE. Numbers in parentheses indicate number of rats/group.

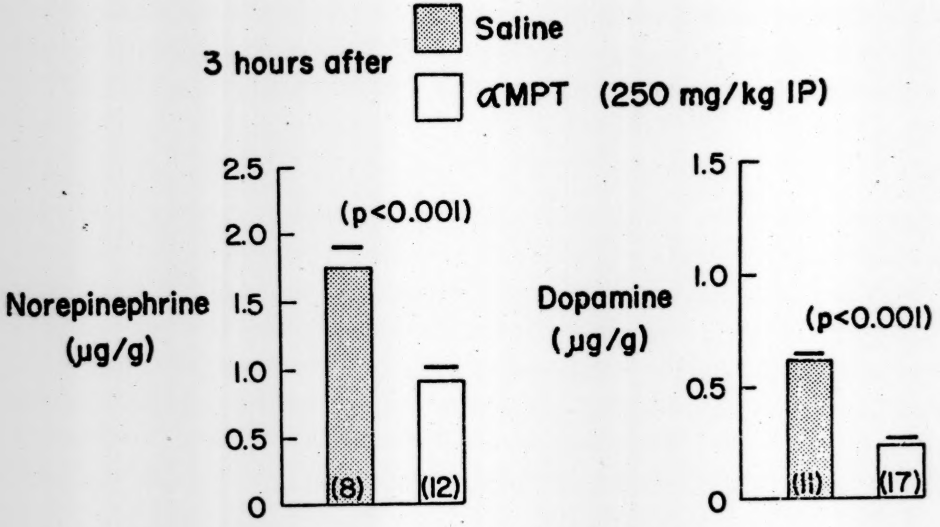
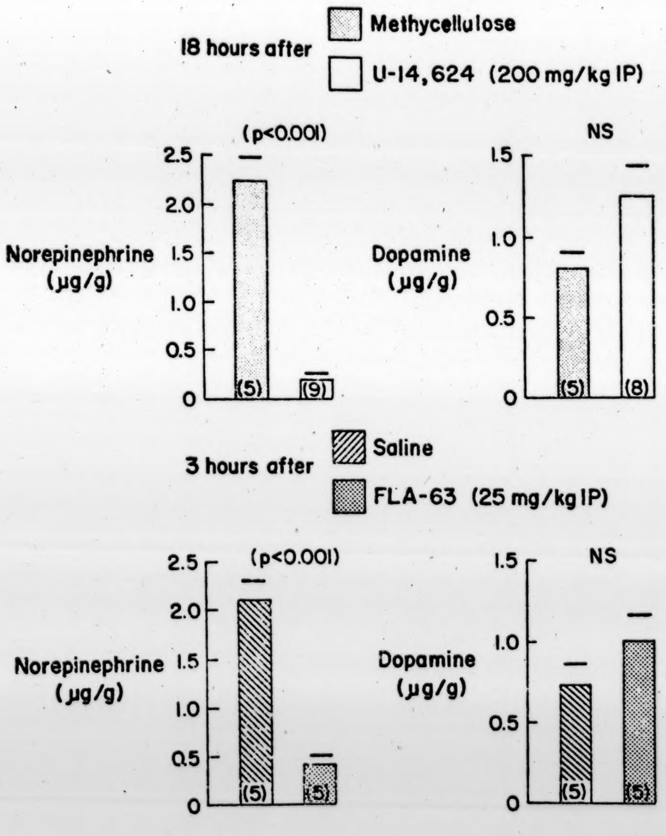


Figure 5. Effect of U-14,624 and FLA-63 on hypothalamic levels of norepinephrine and dopamine in individual ovx rats. Values given represent mean + SE. Numbers in parentheses indicate number of rats/group.



drugs significantly reduced hypothalamic norepinephrine levels and inhibited episodic LH release.

4. U-14,624 and DOPS

To restore episodic LH release in ovx rats treated with U-14,624 (200 mg/kg ip), dihydroxyphenylserine (DOPS) (250 mg/kg ip) was given 18 hours after U-14,624 administration. Although a second injection of DOPS was given 1 hour after the first dose, LH values did not change from control levels during a 3-4 hour bleeding period in 3/5 rats. In two animals, the LH values increased 1½ hours after DOPS administration and remained high for the rest of the bleeding period (2 hours) (Fig.6)

5. U-14,624 and Isoproterenol

An attempt was made to restore episodic LH release in ovx rats treated with U-14,624 (200 mg/kg ip) by giving isoproterenol (12 μg/kg sc) 18 hours after U-14,624 administration. Repeated doses of isoproterenol, with 1 hour intervals, failed to elicit a change in LH secretion at any time during a 2 hour bleeding period in 3/4 rats. Only one animal responded after the second injection, in this case with a 2-fold increase in LH levels.

6. Apomorphine and ET495

Apomorphine (1.5 mg/kg sc) caused a transient (50-60 minutes) but marked decrease in episodic LH release in 10/12 rats (Figure 7). The onset of inhibition was rapid, occurring within minutes, and lasted 50-60 minutes. Blood LH levels then began to return to control values. Similar effects were seen whether the drug was given sc or ip. Apomorphine also caused a stereotyped gnawing behavior pattern, as seen by Anden *et al*. (1967). A lower dose of apomorphine (0.8 mg/kg sc)

Figure 6. Effect of dihydroxyphenylserine (DOPS; 250mg/kg ip) on LH release in an ovx rat pretreated 18 hours earlier with U-14,624 (200 mg/kg ip). Arrows indicate time of DOPS administration.

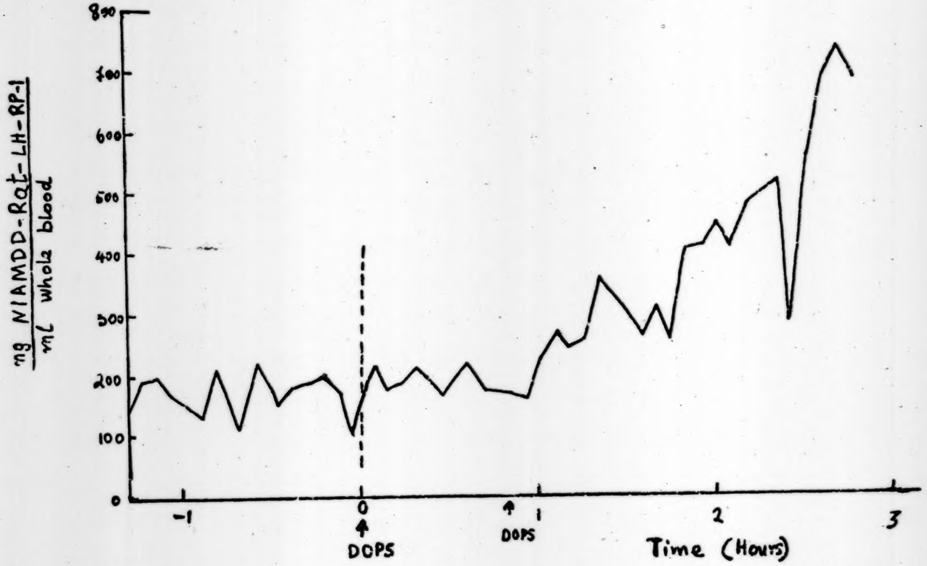
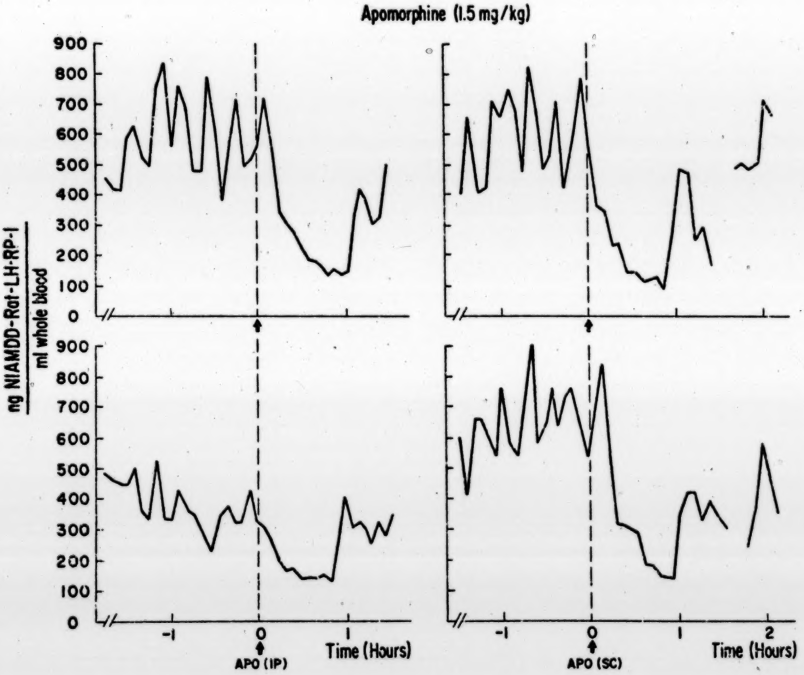


Figure 7. Effect of apomorphine on episodic LH release in individual ovx rats. Apomorphine was given either intraperitoneally (ip) or subcutaneously (sc). Arrows indicate time of apomorphine injection.



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caused a transient inhibition of episodic LH release in 3/5 animals. Injections of saline sc during the course of the bleeding period caused no alterations in episodic LH release in 5/5 rats (Figure 8).

ET495 (20 mg/kg ip) caused a marked decrease in episodic LH release in 6/6 rats (Figure 9). The inhibition began approximately 20 minutes after the injection and lasted 2½-4 hours. ET495 also caused a stereotyped gnawing behavior pattern, as seen following administration of apomorphine. This occurred within minutes and continued for the duration of the experiment. Injections of distilled water (0.2-0.3cc ip) during the bleeding period caused no alteration in episodic LH release in 4/4 rats (Figure 10).

7. Apomorphine or ET495 following Pimozide or Butaclamol

Butaclamol alone:

To test whether endogenous DA plays a role in modulating episodic LH release, animals were bled before and after d- or l-butaclamol. Neither compound altered the mean blood level of LH or the episodic secretion of LH in ovx rats (Figures 11, 12, 13, 14).

Pimozide and Apomorphine; Butaclamol and ET495 or Apomorphine:

To test whether the inhibitory actions of apomorphine and ET495 on LH release were exerted by a specific action on dopamine receptors, ovariectomized rats were pretreated 16 hours before apomorphine with pimozide, and with d-butaclamol 3-4 hours before apomorphine or ET495. In these animals, the stereotyped gnawing behavior seen following apomorphine and ET495 alone was not present. In 6/7 rats, pimozide pretreatment markedly reduced (2) or eliminated (4) the inhibitory effects due to apomorphine on episodic LH release (Figure 15). When

Figure 8. Effect of saline injection (SC) during experimental bleeding period on episodic LH release in an ovx rat. Arrow indicates time of saline injection.

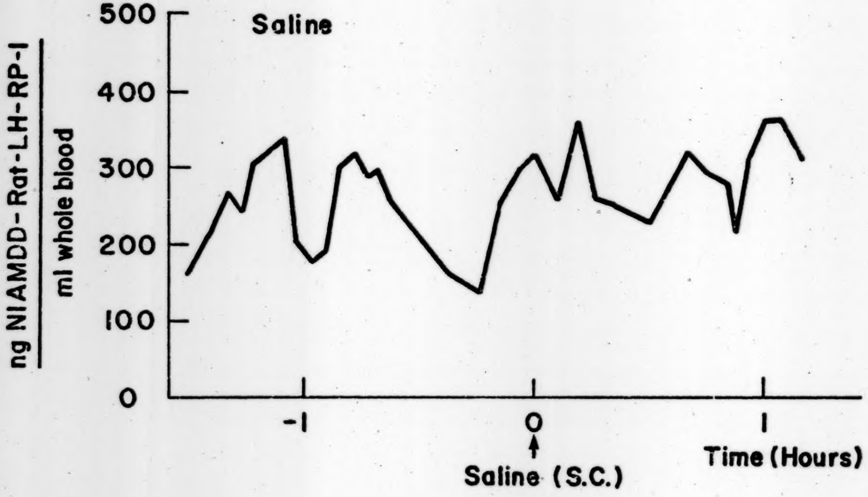

Figure 9. Effect of ET495 (20mg/kg ip) on episodic LH release in individual ovx rats. Arrow indicates time of ET495 administration.

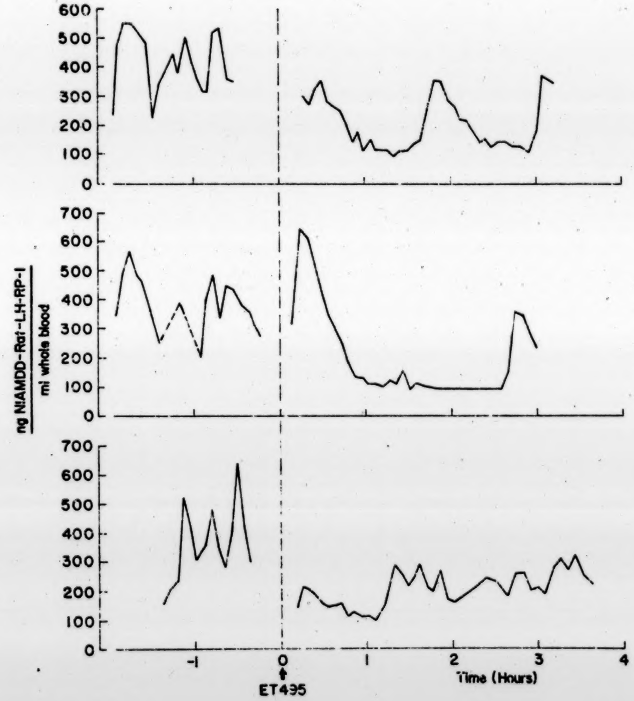

Figure 10. Effect of distilled water injection (ip) during experimental bleeding period on episodic LH release in individual ovx rats. Arrow indicates time of distilled water administration.

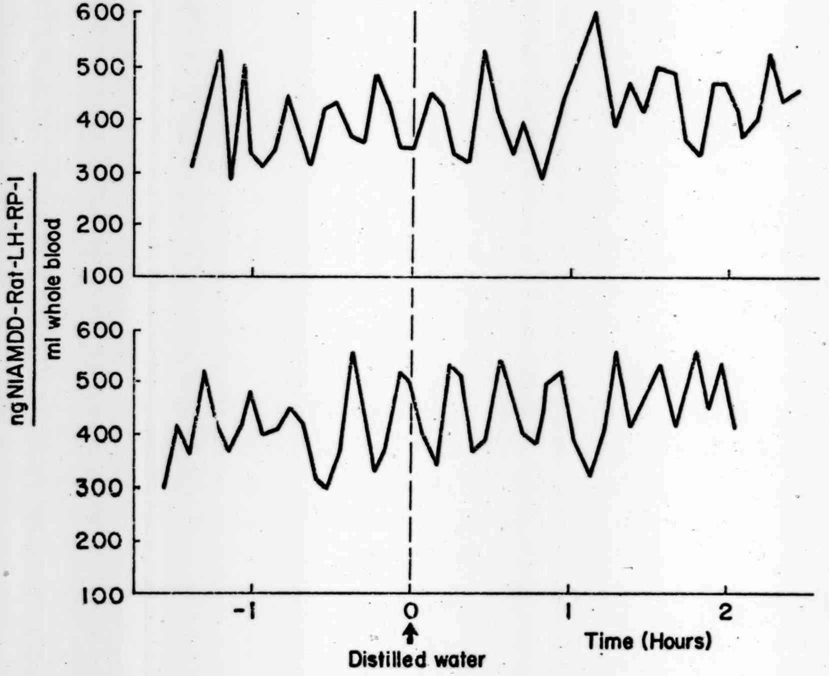

Figure 11. Effect of apomorphine (APO; 1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with d-butaclamol (1mg/kg ip). Arrows indicate time of apomorphine or butaclamol injection.

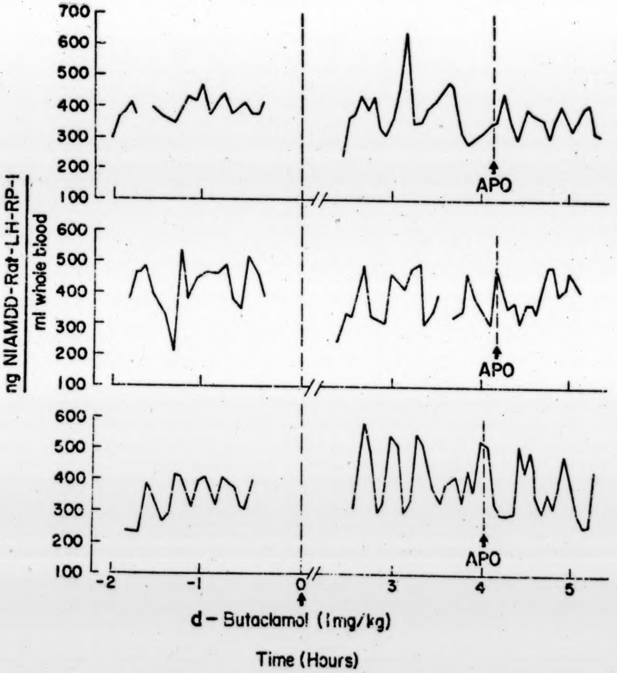

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Figure 12. Effect of ET495 (20mg/kg ip) on episodic LH release in individual ovx rats pretreated 3-4 hours earlier with d-butaclamol (1mg/kg ip). Arrows indicate time of ET495 or butaclamol injection.

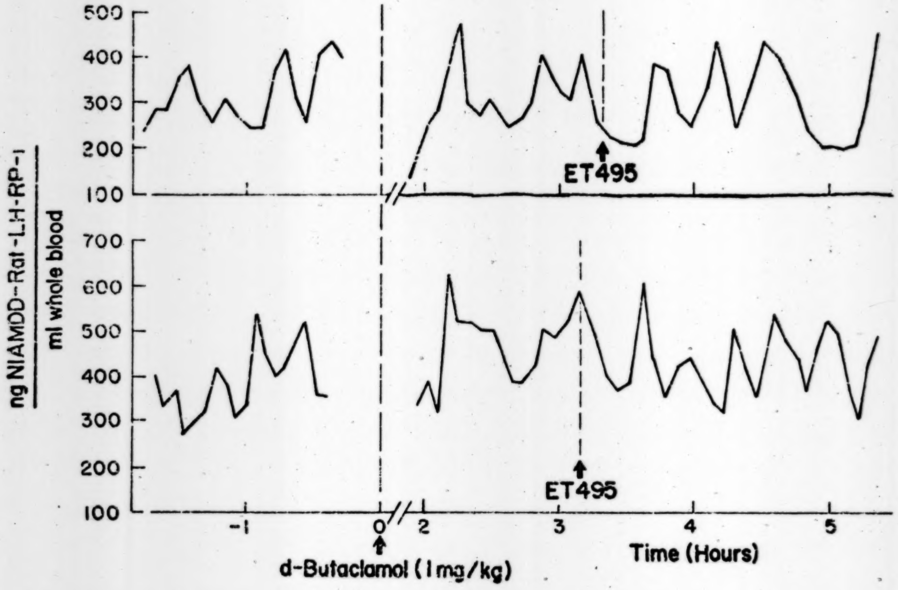


Figure 13. Effect of apomorphine (APO; 1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with d-butaclamol (0.5mg/kg ip). Arrows indicate time of apomorphine or butaclamol injection.

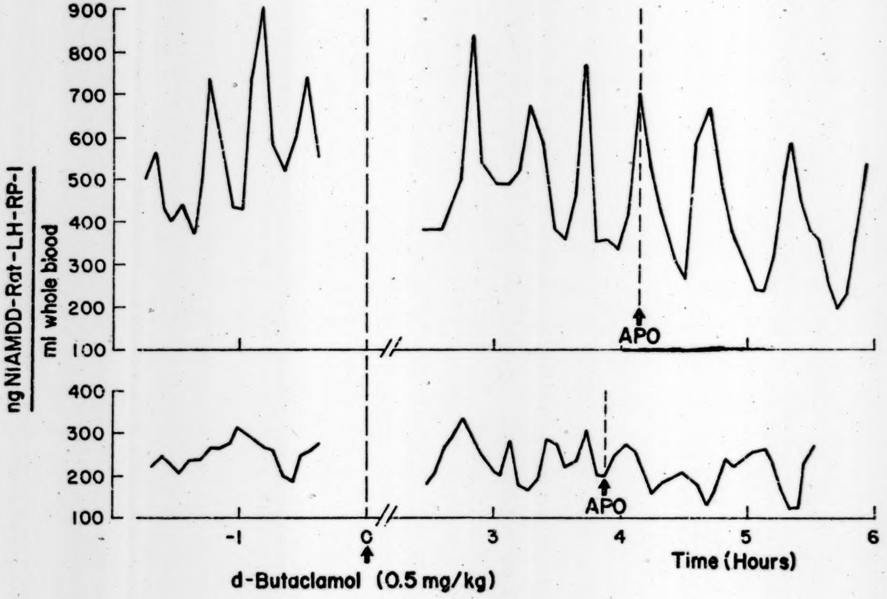


Figure 14. Effect of ET495 (20mg/kg ip) or apomorphine (1.5 mg/kg sc) on episodic LH release in individual ovx rats pretreated 4 hours earlier with l-butaclamol (1mg/kg ip). Arrows indicate time of ET495, apomorphine or butaclamol injection.

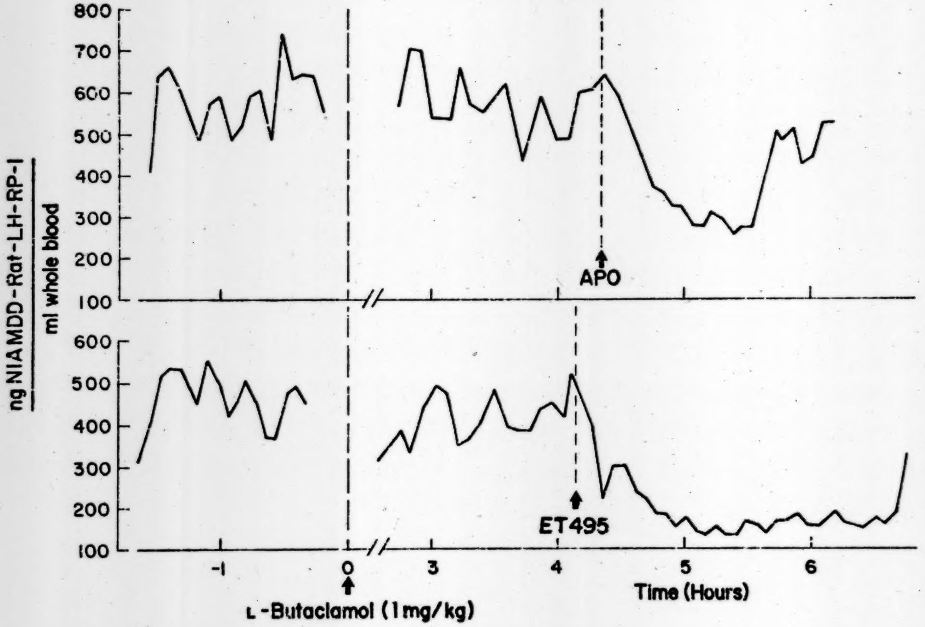
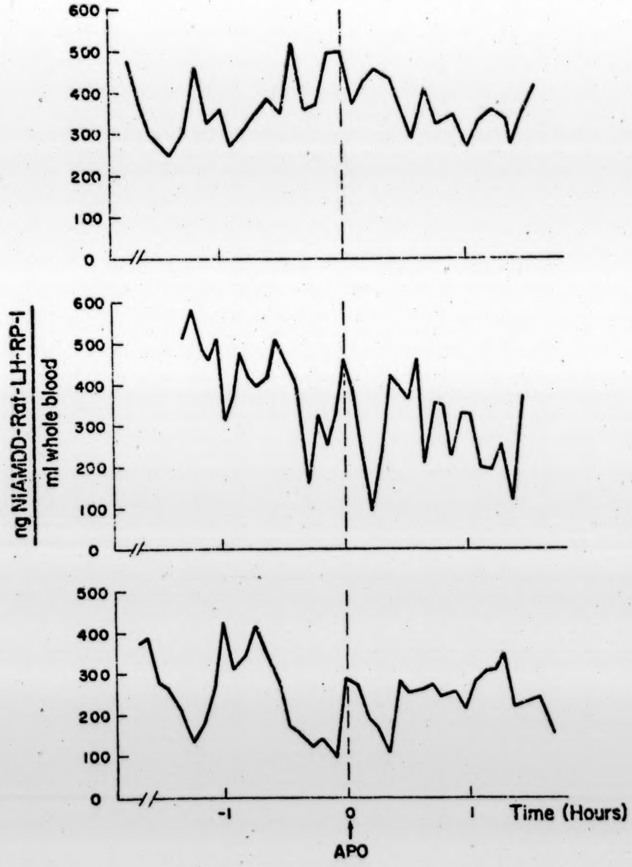


Figure 15. Effect of apomorphine (APO; 1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 16 hours earlier with pimozide (1.26mg/kg sc). Arrow indicates time of apomorphine injection.



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pimozide was given to 5 rats 6 hours prior to apomorphine, it reduced in 2 and eliminated in 1 the inhibitory effects of apomorphine.

Prior to administration of apomorphine, mean blood LH levels in pimozide pretreated rats appeared similar to values obtained in untreated ovariectomized rats. Although blood LH values were not determined prior to administration of pimozide, this compound alone did not seem to alter the mean blood level of LH in ovariectomized rats.

In 4/4 rats, d-butaclamol treatment (1 mg/kg ip) eliminated the inhibitory effects on episodic LH release due to apomorphine (Figure 11), and in 4/4 due to ET495 (Figure 12). When a lower dose of d-butaclamol (0.5 mg/kg ip) was given prior to apomorphine, it eliminated the inhibitory effects of apomorphine in 3/4 rats (Figure 13). L-butaclamol pretreatment (1 mg/kg ip) did not block the inhibitory effects on episodic LH release due to ET495, nor the stereotyped gnawing behavior seen following administration of this compound in 2/2 rats (Figure 14). Similarly, the inhibitory effects of apomorphine on episodic LH release in 2/3 rats and the stereotyped gnawing behavior were still observed despite pretreatment with l-butaclamol (Figure 14).

8. Measurement of plasma corticosterone levels during pharmacological manipulation of DA receptors

Treatment with apomorphine (1.5 mg/kg sc) caused a significant increase of plasma corticosterone above control levels at 20 minutes ($P<0.02$) and 1 hour ($P<0.005$) after its administration.

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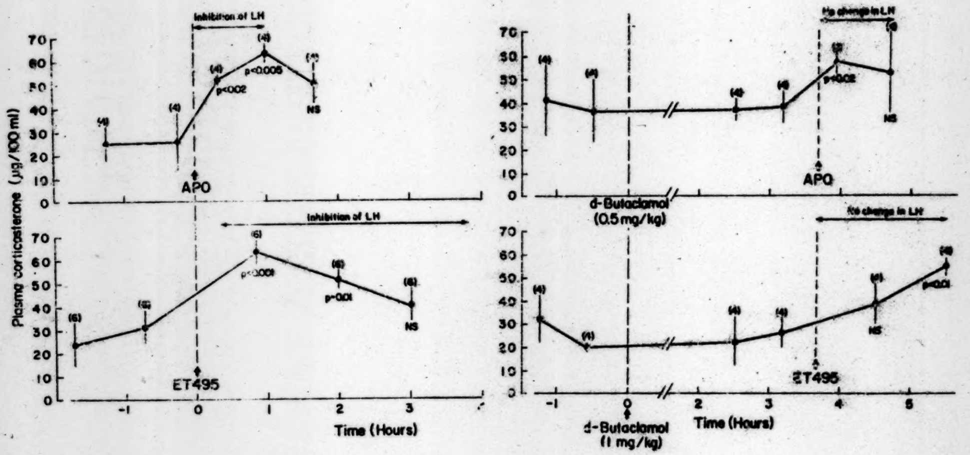
Figure 16. Effect of ET495 or apomorphine alone (left) or after d-butaclamol pretreatment (right) on plasma corticosterone levels in ovx rats. Values given represent mean ± SE. Numbers in parentheses indicate number of rats.



Moreover, a significant increase in plasma corticosterone levels above post-butaclamol values was seen at 15 minutes ($P<0.02$) after apomorphine administration in rats pretreated with d-butaclamol. However, there was no change in episodic LH patterns after apomorphine administration in these rats (Figure 16).

Similar results were obtained after the administration of ET495. This agent caused a significant increase in plasma corticosterone levels at 50 minutes ($P<0.001$) and at 2 hours ($P=0.01$) after its administration and a marked inhibition of episodic LH release (Figure 16). There was no significant increase in plasma corticosterone levels 3 hours after ET495, yet the inhibitory action of this compound on LH release still occurred. Moreover, pretreatment with d-butaclamol prevented the inhibitory action of ET495 on LH release but a significant increase in plasma corticosterone levels above post-butaclamol values ($P<0.01$) was still observed at 1½ hours after ET495 (Figure 16). Therefore, no correlation was observed between the elevation of plasma corticosterone levels and the inhibitory action of these compounds on LH release.

9. PCPA and Apomorphine

To test a possible interaction between central nervous system dopaminergic and sertoninergic neurons on episodic LH release, ovariectomized rats were pretreated 72 hours before apomorphine with PCPA, an inhibitor of serotonin synthesis. In these animals, the stereotyped gnawing behavior seen following apomorphine alone was still observed. In 5/5 rats, apomorphine caused a marked decrease in episodic LH release. The inhibition, occurring within minutes was present for 1-2 hours after apomorphine injection (Figure 17).

II Neurotransmitter infusions in the III ventricle

1. Artificial cerebrospinal fluid (CSF)

Infusion of artificial CSF containing 0.1% ascorbic acid, at a rate of 25λ/h during the bleeding period, caused no effect in episodic LH release in 5/6 rats (Figure 18).

2. Dopamine-HCl (DA)

Prior to infusion of DA, normal fluctuations in LH release were observed. Dopamine HCl (2.5 μg/h) caused a prolonged interval (35-50 minutes compared to 20-30 minutes before infusion) between two successive peaks of LH release during the infusion period in 2/4 rats. A higher dose of DA (5 μg/h) caused a transient inhibition (55 minutes) in episodic LH release in 2/4 rats during the infusion period. With an even higher dose (10 μg/h) the inhibition occurred in 6/10 rats (Figure 19). The onset of inhibition occurred with 20-30 minutes and lasted for the duration of the infusion period in 3 rats and in 3 animals continued after the infusion period. Dopamine was also effective in causing behavioral activation, i.e. exploratory, chewing and feeding behavior, at all doses tested.

3. Dopamine plus d-Butaclamol

To test whether the inhibitory effect of DA infusion was exerted by an action on DA receptors found in the brain area around the third ventricle, ovx rats were pretreated 4-5 hours before DA infusion

Figure 17. Effect of apomorphine (1.5mg/kg sc) on episodic LH release in individual ovx rats pretreated 70 hours earlier with pCPA (32mg/100g bwt ip). Arrow indicates time of apomorphine injection.

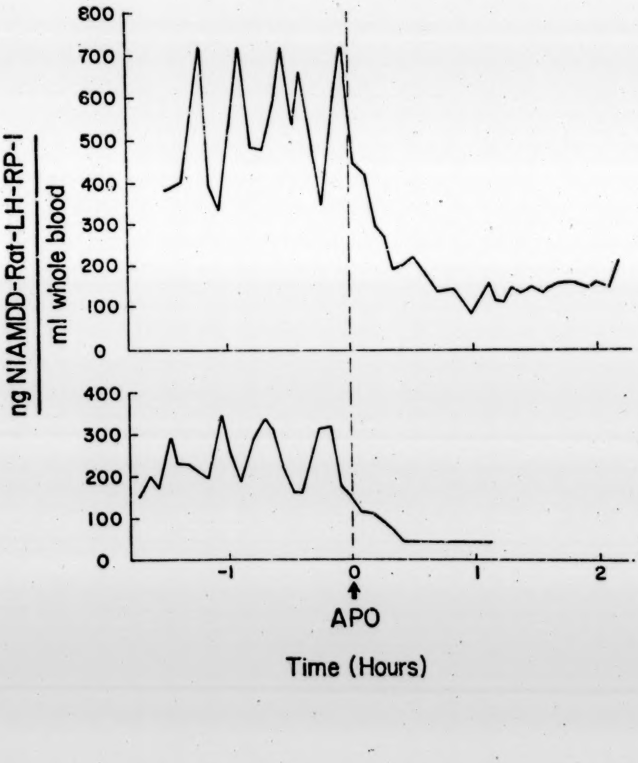


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Figure 18. Effect of cerebrospinal fluid (25λ containing 1λ 0.1% ascorbic acid) infusion into the third ventricle, on episodic LH release in individual ovx rats. Arrows indicate the period of infusion.

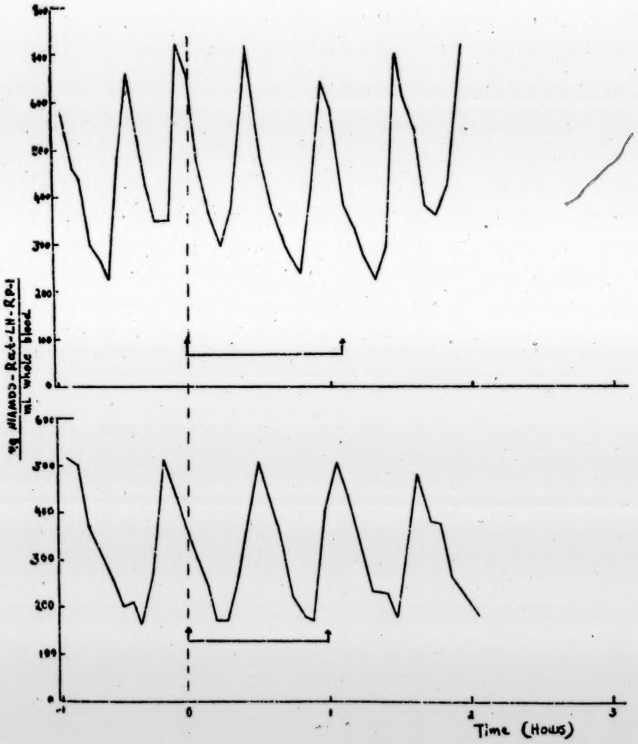
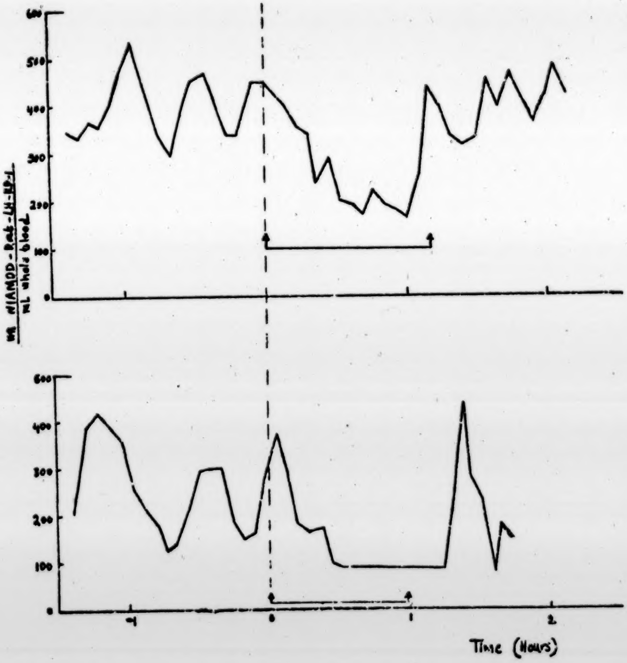


Figure 19. Effect of dopamine-HCl (10μg/25λCSF/h) infusion into the third ventricle on episodic LH release in individual ovx rats. Arrows indicate the period of infusion.



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(10 µg/h) with d-butaclamol. The inhibitory effect of DA on episodic LH release occurred in 3/7 rats (Figure 20) and was absent in the remaining 4 animals.

4. 1-Norepinephrine bitartrate (NE) in rats pretreated with U-14,624

An attempt was made to restore episodic LH release in ovx rats treated with U-14,624 (200 mg/kg ip), a compound previously shown to inhibit norepinephrine synthesis and pulsatile LH release. Several doses of norepinephrine were infused 18 hours after U-14,624 administration. At all doses tested, norepinephrine failed to elicit a change in LH secretion at any time during the infusion period or after. The LH values remained the same as during the pre-infusion control period in 3/3 rats infused with 10 µg NE/h, in 4/4 rats infused with 20 µg NE/h and in 2/2 rats infused with 50 µg NE/h (Figure 21).

5. 1-Norepinephrine bitartrate (NE) in untreated ovariectomized rats

Infusion of several doses of NE caused a transient but marked inhibition of LH release during the infusion period. The onset of inhibition occurred within 10-20 minutes and lasted in 3 animals for the duration of the infusion period and in 8 animals for varying times after the infusion. Blood LH levels then began to return to control values. This effect occurred in 1 out of 2 rats with 2.5 µg NE/h, 2/2 rats with 5 µg NE/h, 4/4 rats with 10 µg NE/h, in 4/4 with 20 µg NE/h (Figure 22). Lower doses of NE (1 µg and 0.5 µg/h) had no effect in 5/5 rats. Norepinephrine was also effective in causing behavioral activation, i.e. exploratory, chewing and feeding behavior

Figure 20. Effect of dopamine-HCl (10μg/25λCSF/h) infusion into the third ventricle on episodic LH release in individual ovx rats pretreated 4-5 hours earlier with d-butaclamol (1mg/kg sc). Arrows indicate time of d-butaclamol administration or the period of DA infusion.

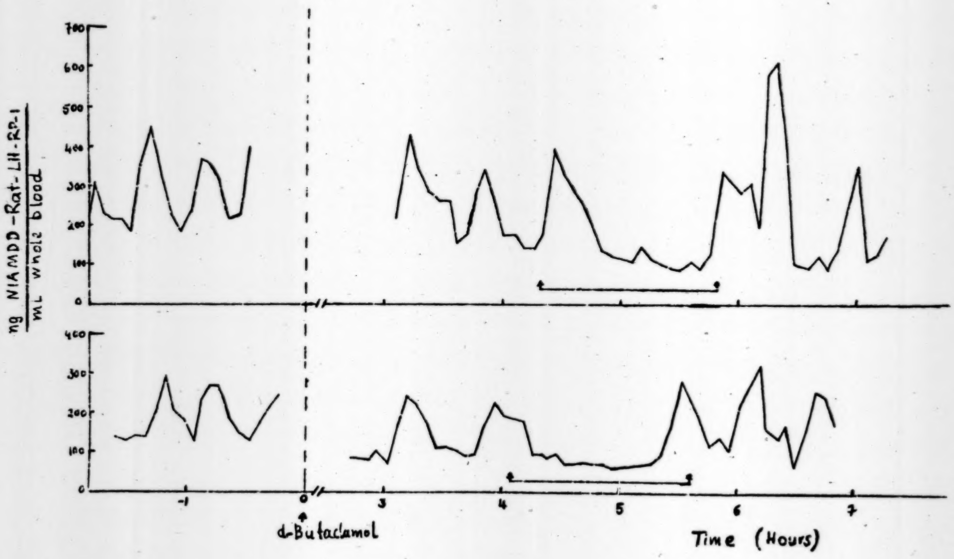


Figure 21. Effect of 1-norepinephrine bitartrate (10,20 or 50μg/25λ CSF/h) infusion into the third ventricle on LH release in individual ovx rats pretreated 18 hours earlier with U-14,624. Arrows indicate the period of infusion.

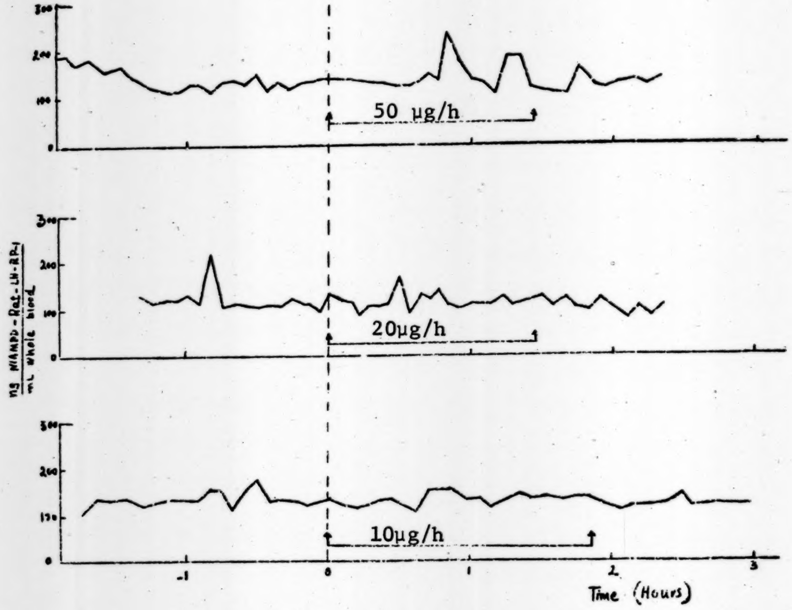
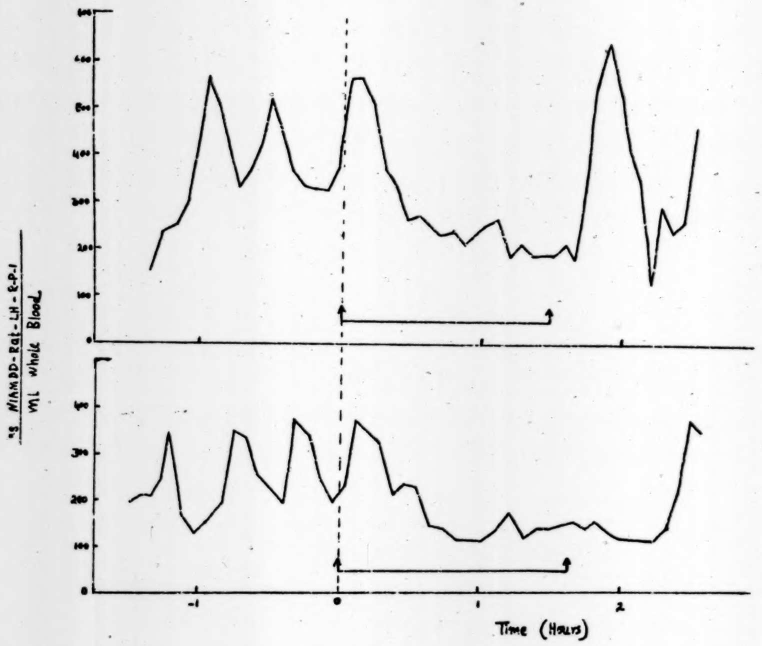


Figure 22. Effect of l-norepinephrine bitartrate (20μg/25λCSF/h in the top and 10μg/25λCSF/h in the bottom) infusion into the third ventricle, on episodic LH release on individual ovx rats. Arrows indicate the period of infusion.



at all doses tested.

6. Serotonin

In 4/5 rats, infusion of serotonin (10 µg/h) caused a transient inhibition after the infusion period. This began 15 minutes after the infusion and had a duration of 30-50 minutes (Figure 23). A lower dose of serotonin (5 µg/h) caused a marked inhibition of episodic LH release in 2/4 rats only during the infusion period with pulsatile LH release returning to control values after the infusion period. No effects on LH release occurred in the other animals during or after the infusion of either dose of serotonin.

III Estrogen administration

In order to examine any possible interaction between estrogen and catecholamines in episodic LH release, ovx rats were primed with 5 µg estradiol benzoate/100g bw/day sc for 2 days prior to peripheral administration of dopamine receptor stimulating agents, or central administration of 1-norepinephrine into the III ventricle.

1. Estrogen and apomorphine or ET495

When compared to nonsteroid treated rats, estrogen markedly reduced blood LH levels and inhibited episodic LH release in 7/7 rats. This is in agreement with the finding of Diekman and Malven (1973), Yamaji et al. (1972), and Blake et al. (1974). Although the levels of LH were low, the administration of apomorphine (1.5 mg/kg sc) failed to cause any further inhibition of LH release in 3 rats (Figure 24) and produced a short pulse (25% increase) of LH release 8 minutes after its administration which lasted for 30 minutes in

Figure 23. Effect of serotonin (10μg/25λ CSF/h) infusion into the third ventricle, on episodic LH release in individual ovx rats. Arrows indicate the period of infusion.

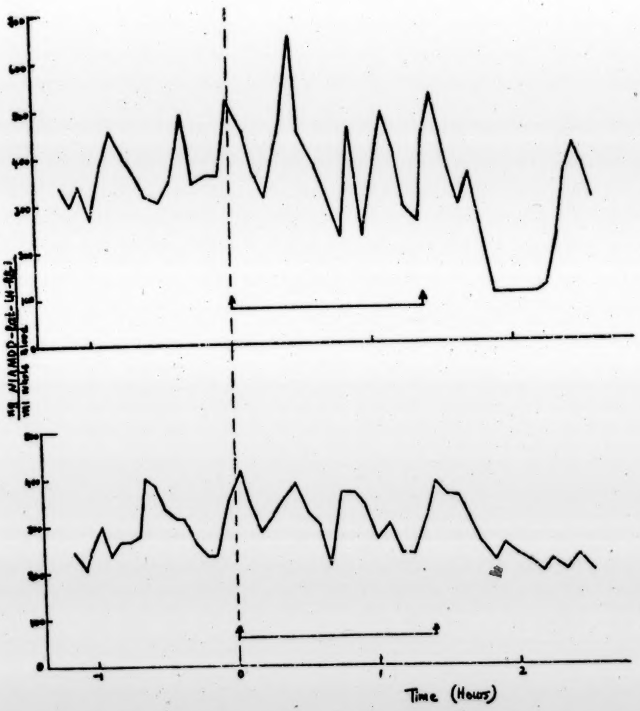
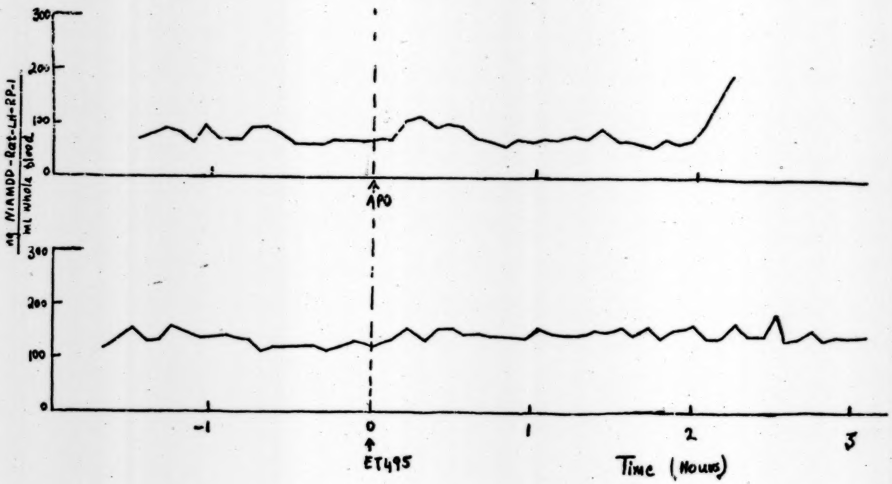


Figure 24. Effect of apomorphine (APO; 1.5mg/kg sc) or ET495 (20mg/kg ip) on LH release in individual ovx rats primed for two days prior with estradiol-benzoate (5µg/100g bw/day sc in oil). Arrows indicate time of apomorphine or ET495 injection.



1 rat. The administration of ET495 failed to alter the blood levels of LH in 3/3 rats (Figure 24).

2. Estrogen and norepinephrine

Estrogen markedly reduced blood LH levels and inhibited episodic LH release in 6/6 rats. Infusion of norepinephrine bitartrate into the third ventricle caused increases in LH release in one out of two rats with 5 μg/h (Figure 25, bottom), in two out of two rats with 10 μg/h (Figure 25, middle), and in one out of two rats with 20 μg/h (Figure 25, top).

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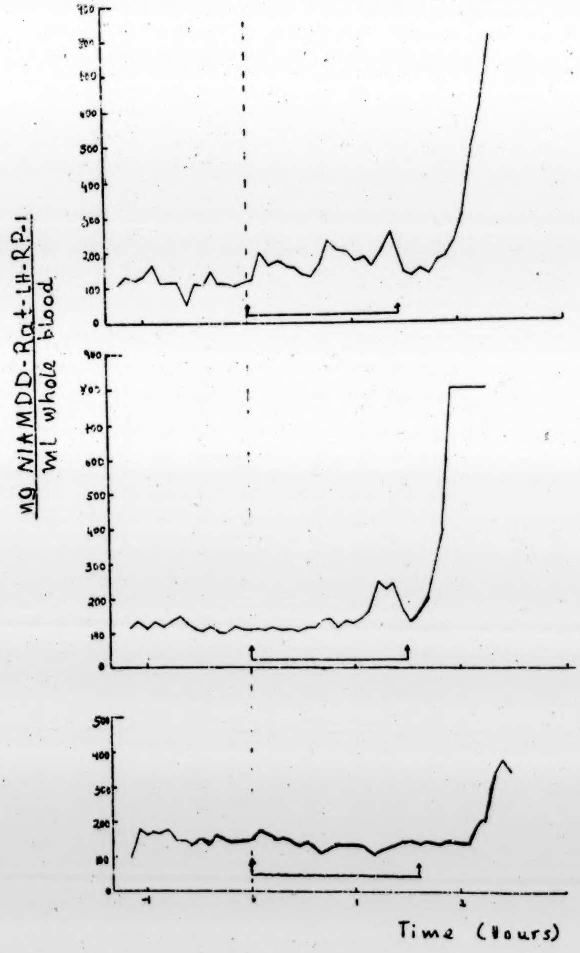
Figure 25. Effect of l-norepinephrine bitartrate (5, 10, 20μg/25λCSF/h) infusion into the third ventricle on LH release in individual ovx rats primed for two days prior with estradiol benzoate (5mg/100g bw/day sc in oil). Arrows indicate the period of infusion.



DISCUSSION

Various studies have suggested that the brain may play a significant role in the mechanism underlying episodic LH release (Bhattacharya, *et al.*, 1972; Knobil 1974; Blake, *et al.*, 1974; Osland, *et al.*, 1975; Carmel, *et al.*, 1976; Bledsoe and Ramirez 1976). Pharmacological approaches to that question have suggested catecholamine involvement in this process. Bhattacharya, *et al.* (1972) have reported that a-adrenergic blocking agents (phenoxybenzamine and phentolamine) and neuroleptic agents (haloperidol and chlorpromazine) blocked episodic LH release in the ovx monkey. However, episodic LH release persists in ovx monkeys (Krey, *et al.*, 1975) and, in certain cases, rats (Blake and Sawyer 1974) following complete deafferentation of the MBH, while with the same procedure the norepinephrine content of the deafferented hypothalamines is depleted (Weiner, *et al.*, 1972). This would suggest that NE may not play a role in this pulsatile pattern of LH release. Moreover, recent studies (Gallo 1976) suggest a possible involvement of serotonin in the inhibitory effect on episodic LH release produced by stimulation of the arcuate nucleus in the hypothalamus.

In the present experiments, several pharmacological agents were used to investigate further possible monoamine involvement in the modulation of pulsatile LH release in the ovx rat. Selective depletion of norepinephrine by β-hydroxylase inhibitors administered peripherally (U-14,624 or FLA-63) blocked LH release, and depleted hypothalamic norepinephrine. However, αMPT significantly depleted both HNE and hypothalamic dopamine, yet failed to alter episodic LH release. These data suggest that norepinephrine could have a stimulatory effect in the neural events controlling episodic LH release, since selective depletion of NE blocked this process. The failure of αMPT to alter pulsatile LH release may be explained by the greater depletion of HNE after U-14,624 administration than after αMPT. Alternately, αMPT depleted both HNE and HDA, and the functional ratio of the remaining catecholamines may have been capable of activating the LH-RH discharge that precedes episodic LH release. Cocchi, et al. (1974) also found that αMPT was ineffective in diminishing the postcastration rise in LH release in immature rats, although FLA-63 eliminated the postcastration increase in plasma LH in those animals. Although Ojeda and McCann (1973) found that αMPT prevented the postcastration increase in plasma LH concentration in male rats, others (Donoso, et al., 1971) indicate that this agent does not have this effect in chronically ovx rats.

However, αMPT given to intact rats on the day of proestrus blocked ovulation. The administration of U-14,624 18 hours prior to the critical period of LH release had the same effect. Although we did not measure HNE and HDA in that experiment, it is possible that the neural events triggering cyclic ovulatory LH release, and pulsatile LH release in ovx rats, may differ.

Episodic LH release has been reported to persist in the ovx rat (Blake and Sawyer 1974) and monkey (Krey, *et al.*, 1975) after surgical interruption of all neural connections to the MBH. Since this procedure causes total depletion of NE in the MBH (Weiner, *et al.*, 1972) these latter reports would suggest that NE may not play a role in episodic LH release and would, therefore, not support the possibility that NE may be an excitatory neurotransmitter in episodic LH release. However, if persistent diestrus develops following deafferentation, then after ovariectomy blood LH levels will be low and episodic LH release absent (Blake and Sawyer, 1974). Moreover, it is only in rats showing persistent diestrus after deafferentation that total depletion of norepinephrine (Weiner, *et al.*, 1972) is correlated with the absence of pulsatile LH release. Based on anatomical studies (Ungerstedt, 1971a), however, we would expect that the content of norepinephrine in the deafferented MBH would be reduced equally regardless of whether the rat demonstrated persistent estrus or diestrus, although this point has not been studied. Furthermore, surgical isolation of this MBH tissue removes not only noradrenergic input to the island, but all other connections as well. Though greatly decreased some luteinizing hormone-releasing hormone (LH-RH) remains within the MBH following deafferentation (Weiner, *et al.*, 1975). In addition to removing a possible excitatory noradrenergic input, deafferentation might also eliminate an inhibitory input to these remaining LH-RH containing neurons. If these LH-RH neurons are spontaneously active, this could explain the maintenance of episodic LH release, at least in rats made persistent estrus by hypothalamic deafferentation.

An attempt was made to restore episodic LH release in rats pretreated with U-14,624, by administration of DOPS, a precursor of NE, or by giving isoproterenol, a β-adrenergic receptor agonist. Neither of these drugs was effective in restoring the rhythm. Moreover, Kalra and McCann (1974) have reported that DOPS only partially restored the blockade of the ovulatory surge of LH and ovulation produced by DDC, a β-hydroxylase inhibitor, or αMPT. A possible explanation is that the hypothalamic capillaries in the rat have high concentrations of l-aromatic amino acid decarboxylase which would result in the conversion of DOPS to NE in the endothelial cells and would limit the uptake of the amine by neural elements (De la Torre, 1972). The ineffectiveness of isoproterenol in restoring the episodic LH pattern may indicate that the possible excitatory role of NE is not mediated by a β-adrenergic receptor. Moreover, the previous literature suggesting NE as an excitatory neurotransmitter in LH release and episodic LH release indicates it works through an a-adrenergic receptor, not a β receptor (Kalra, _et al_., 1972; Knobil 1974).

Biochemical and pharmacological studies suggest an interaction between serotonergic and catecholaminergic neurons in the brain. Johnson, _et al_. (1971) have reported elevated brain

UMI

serotonin levels and 5 HIAA after administration of β-hydroxylase inhibitors. However, it does not appear that the inhibitory effect of U-14,624 is exerted through elevated serotonin levels since episodic LH release is inhibited at 18 hours after U-14,624, at a time when serotonin levels are close to normal (Johnson, et al., 1971). Moreover, αMPT also increased serotonin levels (Samanin 1975), yet does not alter episodic LH release. However, hypothalamic or brain serotonin levels were not determined in the present studies after pretreatment with β-hydroxylase or tyrosine hydroxylase inhibitors.

The role of DA, as reported in the literature, is not clear. Injections of DA into the third ventricle produced increases in LH-RH activity in stalk plasma of intact rats (Kamberi, et al., 1969) or peripheral plasma of hypophysectomized rats (Schneider and McCann 1970a). This would suggest an excitatory role for DA in the regulation of LH release. However, others have suggested an inhibitory role, based on histochemical fluorescence changes in tuberoinfundibular neurons (Fuxe, et al., 1969; Ahren, et al., 1971; Fuxe, et al., 1972) and on inhibitions by dopamine of LH release from pituitary-median eminence incubations (Miyachi, et al., 1973). Sawyer, et al. (1974) reported that intraventricular administration of dopamine failed to increase LH release in estrogen primed rabbits, but did reduce the excitatory effect on LH release of norepinephrine similarly administered 1-2 weeks after dopamine.

In order to investigate the role of DA in episodic LH release we used drugs acting on DA receptors. Selective stimulation of DA receptors by apomorphine or ET495 blocked pulsatile LH release. Moreover, selective blockade of DA receptors by pimozide or d-butaclamol in doses which do not affect norepinephrine turnover (Andén, et al., 1970; Lippman and Pugsley, 1975) or acetylcholine metabolism (Iversen 1975) prevented the apomorphine or ET495 induced inhibition of episodic LH secretion. However, pretreatment with l-butaclamol, a biologically inactive form of butaclamol (Lippmann, et al., 1975) had no effect. These data suggest that activation of DA receptors can inhibit episodic LH release. However, the inability of pimozide and d-butaclamol alone to alter episodic LH discharge suggests that under physiological conditions, DA may not play a role in the modulation of episodic LH release.

In order to relate any possible stress induced changes in plasma corticosterone levels to the inhibitory action of apomorphine or ET495 on LH release, plasma corticosterone levels were determined. Increases in plasma corticosterone levels were seen both following apomorphine or ET495 alone (when LH release was inhibited) as well as after apomorphine or ET495 administration to d-butaclamol pretreated rats (when LH levels did not change). This would indicate no correlation betwen elevations of plasma corticosterone and inhibitory actions of apomorphine or ET495 on LH release. Blake (1975) has found that, withthe exception of immobilization, a variety of stresses failed to inhibit episodic LH release.

Evidence in the literature suggests a possible interaction between serotonergic and dopaminergic neurons (Cools 1974; Grabowska, et al., 1973). Moreover, apomorphine has been shown to increase brain serotonin levels (Grabowska, et al., 1973a; Simalowska 1974). However, this does not appear to explain the inhibitory effect of apomorphine on episodic LH release since this effect still occurred in ovx rats pretreated with PCPA, a serotonin synthesis inhibitor (Koe and Weissman, 1966). However, serotonin levels were not determined after administration of DA receptor agonists. It would be of interest to examine whether apomorphine can elevate serotonin levels in PCPA treated animals, or if pimozide or d-butaclamol can prevent this effect.

To further examine the roles of various neurotransmitters in eposidic LH release, these substances were infused into the third ventricle of ovx rats. Infusion of DA inhibited episodic LH release in 60% of the animals, but this effect still occurred in 43% of the animals pretreated with d-butaclamol. Infusion of NE in doses of 2.5-20μg/h inhibited pulsatile LH release. Lower doses had no effect. Although other expalanations may be possible these results suggest that DA may exert its inhibitory effect by firstbeing transformed into NE. A second possibility is that systemically administered butaclamol may not reach DA receptors in high enough concentrations to interfere with levels of administered dopamine reaching DA receptors in close approximation to

UMI

the third ventricle. However, indirect evidence suggests that butaclamol crossed the blood-brain barrier since it blocks apomorphine induced sterotyped behavior (Voith and Herr, 1975). In addition, Fuxe, et al. (1975) have shown that DA nerve endings in the ME may lack presynaptic receptors since there is no increase in DA turnover following administration of DA receptor blockers (Fuxe, et al., 1975). Then one should ask: where do apomorphine or ET495 exert their action on LH release? Is their action mediated by extrahypothalamic DA receptors? Which is the DA pathway involved in their action? Further experiments are necessary to answer these questions.

The inhibitory effect on episodic LH release seen during NE administration into the third ventricle suggests that NE can exert an inhibitory role, possibly by stimulating adrenergic receptors located around the third ventricle region which have an inhibitory influence in this process. Further studies are needed to define the type of adrenergic receptor involved. This finding is contradictory to the effects on episodic LH release obtained after peripheral administration of β-hydroxylase inhibitors. It is possible that U-14,624 and FLA-63 exert their action by affecting different populations of noradrenergic neurons throughout the whole brain, while NE infusion into the third ventricle affects only regions in which there may be adrenergic receptors with inhibitory influences on LH-RH neurons. Although norepinephrine has been suggested to play an excitatory role in the neural events controlling the release of LH (Sawyer, et al.,

UMI

1947; Sawyer, *et al.*, 1949; Kalra, *et al.*, 1972; Ojeda and McCann 1973; Kalra and McCann 1973; Sawyer, *et al.*, 1974, Cocchi, *et al.*, 1974), Kobayashi and Matzui (1969) have reported that when norepinephrine implants were placed in the ME in cycling rats, the rats showed irregular estrous cycles with prolonged estrous and diestrous phases and decreased ovarian weights. Control cholesterol implants had no effect. NE implants in the arcuate nucleus induced weaker effects and implants in the adenohypophysis or in hypothalamic nuclei other than the arcuate nucleus had no effect. Implantation of DA provoked effects that were similar to those of NE on the estrous cycle and ovarian weight. Donovan and Harris (1956) and Kamberi, *et al.* (1970) found that slow injection to NE into the rabbit and rat adenohypophysis had no effect on LH release. Therefore, the effects of NE implanted in the ME or arcuate nucleus are probably not exerted directly on the adenohypophysis. The possibility that NE reduces the amounts of LR-RH reaching the pituitary by constricting the portal vessels, seems unlikely since the NE implants did not affect other endocrine glands (e.g. adrenal-thyroid) (Kobayashi and Matzui 1969). Moreover, Cramer and Porter (1973) reported that ventricular injections of norepinephrine appear to inhibit LH release in castrated animals. Moreover, intraventricularly administered dopamine inhibited the release of LH normally seen following intraventricular injections of 0.15 M NaCl in castrated male rats.

UMI

The inability of NE infusion into the third ventricle to restore the LH rhythm in U-14,624 pretreated rats may then be due to its possible inhibitory effect on the LH-RH neurons in this region. The NE levels in the rest of the brain regions in U-14,624 treated animals are still depleted and episodic LH release is not restored.

Finally variable effects were seen during infusion of serotonin. This transmitter either inhibited episodic LH release during or even after the infusion period, or had no effect. Although 5HT has been suggested to be an inhibitory neurotransmitter in LH release (Schneider and McCann 1970; O'Steen 1965; Kordon 1971; Labhsetwar 1971), from the infusion data we cannot conclude any definite role for this substance.

To examine any possible interaction between estrogen and catecholamines in episodic LH release, ovx rats were primed with estrogen prior to the infusion of NE into the third ventricle, or to peripheral administration of DA receptor agonists. Infusions of NE increased the levels of LH in most of the estrogen primed animals, the increase being most evident after the infusion period. The administration of DA receptor agonists failed to alter the blood levels of LH. This would suggest that the presence of estrogen may be capable of reversing the inhibitory effect of NE on episodic LH release in ovx rats, or that NE can accelerate a positive feed-back action of estrogen in these animals. Recently, Krieg and Sawyer (1976) reported that in long-term ovx, estrogen-progesterone-primed animals, intraventricular infusion of NE induced significant rises in plasma LH levels. Gallo and Osland (1976) have reported that electrical stimulation of arcuate nucleus in ovx rats inhibited episodic LH release and excited LH secretion after pretreatment of ovx animals with estrogen. Thus, the presence of estrogen can modify the neural substrate involved in the regulation of episodic LH release. Further studies attempting to clarify the role of a possible interaction between estrogens and catecholamines need to be done.

In conclusion, the above data pose several interesting questions. 1) Are there noradrenergic neurons in different brain regions with different influences on LH-RH neurons? 2) If so, what are the respective roles of these neurons in the control of episodic LH release? 3)Do they have any interaction with other monaminergic systems in the control of LH release? 4) Do these postulated noradrenergic neurons behave differently under different hormonal (i.e. estrogen) environments? 5) Where does estrogen exert its effect? 6) Does this steroid change the sensitivity of the receptors? The answers to these questions will come from further experimentation.

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